Basic amino acids as modulators of an O-linked glycosylation signal of the herpes simplex virus type 1 glycoprotein gC: functional roles in viral infectivity

Kristina Mårdberg2, Kristina Nyström2, Mads Agervig Tarp1, Edward Trybala1, Henrik Clausen1, Tomas Bergström2, and Sigvard Olofsson1,2

2Department of Virology, University of Göteborg, Guldhedsatan 10B; S-413 46 Göteborg, Sweden; and 1Faculty of Health Sciences, School of Dentistry, Norre Alle 20 DK-2200 N, Denmark

Received on September 22, 2003; revised on February 2, 2004; accepted on March 5, 2004

The herpes simplex virus type 1 (HSV-1) glycoprotein gC-1 is engaged both in viral attachment and viral immune evasion mechanisms in the infected host. Besides several N-linked glycans, gC-1 contains numerous O-linked glycans, mainly localized in two pronase-resistant clusters in the N-terminal domain of gC-1. In the present study we construct and characterize one gC-1 mutant virus, in which two basic amino acids (114K and 117R) in a putative O-glycosylation sequence were changed to alanine. We found that this modification did not modify the N-linked glycosylation but increased the content of O-linked glycans considerably. Analysis of the O-glycosylation capacity of wild-type and mutant gC-1 was performed by in vitro glycosylation assays with synthetic peptides derived from the mutant region predicted to present new O-glycosylation sites. Thus the mutant peptide region served as a better substrate for polypeptide GalNAc-transferase 2 than the wild-type peptide, resulting in increased rate and number of O-glycan attachment sites. The predicted increase in O-linked glycosylation resulted in two modifications of the biological properties of mutant virus—that is, an impaired binding to cells expressing chondroitin sulfate but not heparan sulfate on the cell surface and a significantly reduced plaque size in cultured cells. The results suggested that basic amino acids present within O-glycosylation signals may down-regulate the amount of O-linked glycans attached to a protein and that substitution of such amino acid residues may have functional consequences for a viral glycoprotein involving virus attachment to permissive cells as well as viral cell-to-cell spread.

Key words: attachment/heparan sulfate/O-linked/plaque size

1To whom correspondence should be addressed; e-mail: sigvard.oloftsson@microbio.gu.se

Introduction

The herpes simplex virus type 1 (HSV-1)-specific glycoprotein gC-1 is present in practically all clinical HSV-1 isolates and plays important roles during establishment of HSV infection in humans despite the fact that this glycoprotein is dispensable for viral replication in cell culture (Hidaka et al., 1990; Liljqvist et al., 1999; Olofsson, 1992). Thus gC-1, together with another HSV-1 glycoprotein, gB-1, is responsible for viral binding to cell surface heparan sulfate, which is the primary viral receptor (Gerber et al., 1995; Herold et al., 1991; Herold and Spear, 1994). Moreover, gC-1 is exclusively responsible for viral binding to an alternative receptor of glycosaminoglycan nature, that is, chondroitin sulfate (Mårdberg et al., 2002). Finally, gC-1 constitutes a receptor for factor C3b of the complement system, thereby contributing to HSV-1 immune evasion mechanisms (Friedman et al., 1986, 1996).

Being a highly glycosylated protein, gC-1 contains nine N-linked glycosylation sites of which at least eight are utilized and equipped with complex type glycans exclusively, ranging from di- to tetraantennary structures (Olofsson et al., 1999; Rux et al., 1996). In addition, gC-1 contains numerous O-linked glycans, most of which are localized in two pronase-resistant clusters in the N-terminal part of gC-1 (Biller et al., 2000; Dall’Olio et al., 1985; Olofsson, 1992). One possible function of these O-linked glycans is to contribute to an extended shape of gC-1, thereby facilitating contacts between virion-associated gC-1 and its different ligands (Stannard et al., 1987). The clustered O-linked glycans of gC-1 may also have other functions, including direct glycoepitope signaling to effectors of the immune system. In this context it is intriguing that another herpes virus, for instance, bovine herpes virus type 4, encodes a virus-specific glycosyltransferase, β1,6-N-acetylglucosaminyltransferase (core 2 transferase), which is engaged in formation of immunologically active selectin receptors of O-linked glycan nature (Markine-Goriaynoff et al., 2003; Vanderplasschen et al., 2000). Finally, the O-glycosylated stretches of gC-1 in the vicinity of, say, the heparan sulfate-binding domain (Mårdberg et al., 2001) may affect its binding specificity.

The biosynthesis of O-linked glycans is initiated by the addition of N-acetylgalactosamine (GalNAc) moieties to serine and threonine residues of a completely translated and folded polypeptide chain, a reaction catalyzed by 1 of at least 13 distinct cellular UDP-GalNAc:polypeptide GalNAc-transferases, which are localized throughout the Golgi apparatus (Hassan et al., 2000a). These GalNAc-transferase isofoms have different but partly overlapping peptide acceptor substrate specificities, and they are differentially expressed in cells and tissues (Gruenheid et al., 1993), and gC-1 appears to serve as substrate for multiple human GalNAc-transferases (Biller et al., 2000). This suggests that gC-1 will become O-glycosylated in any cell type included in the broad host cell range of HSV-1.
Here we report that a minor modification of a glycosylation signal of gC-1, that is, substituting alanines for two basic amino acids, resulted in major changes in the extent of O-linked glycosylation. Moreover, this modification changed the biological properties of mutant virus in two different ways, for instance, altered pattern of adsorption to primary glycosaminoglycan receptors and altered plaque morphology, indicating differences in the complex process of viral cell-to-cell spread.

**Results**

**Glycosylation status of gC-1 from HSV-1 with a modified O-glycosylation signal**

During screening of HSV-1 mutants with modified gC-1, where alanine was substituted for basic amino acids, we found one mutant virus (HSV-1gC-1[114K,117R]A) where gC-1 displayed a significantly lower electrophoretic mobility, indicating a significantly larger apparent molecular weight (~8–9 kDa) compared with gC-1 specified by corresponding wild-type virus (HSV-1Rescue) (Figure 1). This difference could not be explained by the mere substitutions themselves because the theoretical peptide molecular weight of mutant gC-1 is in fact lower than wild-type gC-1. Because gC-1 is highly glycosylated it was initially considered conceivable that the mutation interfered with addition or processing of N-linked or O-linked glycans, although the mutations did not alter any of the putative N-glycosylation sites per se. To assess if N-glycan processing was affected mutant and rescue gC-1 were treated with N-glycanase (Figure 1, N-gly lanes). N-glycanase treatment resulted in increased electrophoretic mobilities of the rescue as well as the mutant gC-1, indicating that the enzyme released N-linked glycans from gC-1. However, the difference in electrophoretic mobility mutant and rescue gC-1 remained, demonstrating that differences in the number or composition of N-linked glycans were not responsible for the difference in electrophoretic mobility between gC-1Rescue and gC-1[114K,117R]A.

In contrast to the differences in mobility between completely glycosylated wild-type and mutant gC-1 another picture was observed for the precursor gC-1 (pgC-1) band (Figure 1), which has been demonstrated to contain immature high-mannose N-linked glycans but no O-linked glycans (Biller et al., 2000; Olofsson et al., 1983; Serafini-Cessi et al., 1984; Sommer and Courtney, 1991). Thus mock-treated mutant and wild-type pgC-1 demonstrated identical electrophoretic mobility, which was the case also for N-glycanase-treated (and hence more rapidly migrating) mutant and wild-type pgC-1 bands. The separation into two very close bands of N-glycanase-treated pgC-1Rescue and pgC-1[114K,117R]A, respectively, was occasionally seen and may correspond to pgG with or without the signal peptide sequence present. The results obtained for gC-1 ruled out that possible differences in complex type N-linked glycans were responsible for the high-molecular-weight phenotype of gC-1[114K,117R]A, so the present results suggested that O-linked glycans rather than N-linked glycans accounted for the size difference between gC-1Rescue and gC-1[114K,117R]A.

This was further evaluated by comparing the electrophoretic mobilities of gC-1Rescue and gC-1[114K,117R]A produced in C1300 cells, a mouse neuroblastoma cell line with a general deficiency in galactosyltransferases (Lundström et al., 1987b). Pertinent here is that all O-linked glycans of gC-1 produced in C1300 cells constitute GalNAC mono-saccharides, a few of which are sialylated, which should be compared with a variety of differently sized O-linked glycans up to at least tetrasaccharides that are associated with gC-1 produced in African green monkey kidney (GMK) cells (Lundström et al., 1987a,b). There was no demonstrable difference in electrophoretic mobility between gC-1Rescue and gC-1[114K,117R]A produced in C1300 cells (Figure 2), confirming that the difference in electrophoretic mobility indeed was caused by a higher content of O-linked glycans in gC-1[114K,117R]A. In C1300 cells, the possible extra O-linked glycosylation of...
gC-1(114K,117R)A was obviously insufficient to induce a corresponding shift for the gC-1 variants produced in C1300 cells. In addition, this result suggested that the difference in electrophoretic mobility between gC-1Rescue and gC-1(114K,117R)A, produced in GMK cells, reflected not only a larger number of but also physically larger O-linked glycans of mutant gC-1.

The influence of the gC-1(114K,117R)A mutation on the content of sialic acid in gC-1 was analyzed. Unlabeled glycoprotein extracts from HSV-infected GMK cells were treated with N-glycanase in the presence or absence of sialidase, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subjected to immunoblotting (Figure 3). Two distinct bands were detected for the extracts of each virus: One sharp strong band represented essential unglycosylated pgC-1, owing to N-glycanase treatment, and one broader band representing gC-1 containing O-linked glycans but not N-linked glycans. The strong appearance of the pgC-1 band reflected that the linear epitope used as a target for immunoblotting was partially hidden in completely glycosylated gC-1 (Sjöblom et al., 1992). The sialidase treatment did not result in any consistent and reproducible shift in electrophoretic mobility of gC-1(114K,117R)A or gC-1Rescue.

To further explore possible differences in sialic acid content of gC-1 O-linked glycans, we determined the amount of radioactivity released by sialidase treatment of affinity-purified [3H]-GlcN-labeled gC-1 produced in GMK and C1300 cells, respectively. We have previously shown that gC-1 from C1300 cells does not contain any N-glycan-associated sialic acid, but the materials originating from GMK cells were treated with N-glycanase prior to sialidase treatment to ensure that only sialic acid of O-linked glycans was assayed. The released radiolabeled sialic acid was separated from remaining glycoprotein by Sephadex G25 gel filtration (Figure 4). A small but reproducible difference in the relative content of sialic acid between gC-1Rescue (7.5%–8.0%) and gC-1(114K,117R)A (10.0%–10.5%) was observed, whereas no such difference in the relative sialic acid content was noted for the corresponding gC-1 preparations from GMK cells.

**Prediction of O-glycosylation sites in synthetic peptides by in vitro analysis of the O-glycosylation capacity of multiple human GalNAc-transferase isoforms**

Synthetic peptides representing the O-linked glycosylation signal delimited by amino acids 106 and 124 were analyzed as substrates for individual, purified human GalNAc-transferases (Bennett et al., 1998; Biller et al., 2000). A wild-type peptide (representing rescue virus) as well as a peptide containing the gC-1(114K,117R)A mutations were analyzed with GalNAc-T1, -T2, -T3, or -T6, respectively. The wild-type and the mutant peptide were readily glycosylated by GalNAc-T2, however, the reaction velocity with the mutant peptide was considerably higher than with the wild-type peptide (Table I). The three other enzyme isoforms investigated demonstrated only low catalytic activities with both the wild-type and mutant gC-1 peptide substrates (data not shown).

The difference in velocity of the glycosylation reaction with GalNAc-T2 with the mutant peptide prompted us to assess the final product obtained with GalNAc-T2. Matrix-assisted laser desorption/ionization time-of-flight mass...
spectrometry (MALDI-TOF MS) of the products revealed important differences. There are in total four potential O-glycan sites in the analyzed peptide substrates, and we found that GalNAc-T2 produced GalNAc-glycopeptides after prolonged incubation containing 2 moles of GalNAc with the rescue peptide and 3 moles of GalNAc with the mutant peptide (Figure 5 and Table II). Sites of O-glycan attachments were analyzed by Edman degradation. Initial analysis of number of moles GalNAc incorporated by MALDI-TOF showed GalNAc-T1 and -T2 to produce glycopeptides containing one to two (rescue peptide) and two to three (mutant peptide) GalNAc residues, respectively. The sequencing results, which are presented in Table II, indicated that T111, S115, and T119 of the mutant peptide and that S115 and T119 of the rescue peptide incorporated GalNAc residues. This conclusion is in line with the MS results (Figure 3). Evidence for glycosylation of T110 was found neither for the wild-type nor the mutant peptide.

Modified O-linked glycosylation signal and HSV-1 replication in cell culture

We also determined whether the altered glycosylation influenced HSV-1
gC-1(114K,117R)A replication in cell culture. This was explored on two levels, that is, influence of the mutation on (1) interactions between HSV-1 and the primary receptor, using an *in vitro* assay for attachment to permissive

<table>
<thead>
<tr>
<th>Peptide substrates</th>
<th>GalNAc-T2 activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>gC-1&lt;sub&gt;Rescue&lt;/sub&gt;</td>
<td>200 μM&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PKNNTPAKSGRTKPPGP&lt;sub&gt;124&lt;/sub&gt;</td>
<td>184 (558)</td>
</tr>
<tr>
<td>gC&lt;sub&gt;(114K, 117R)A&lt;/sub&gt;</td>
<td>1466 (3402)</td>
</tr>
<tr>
<td>PKNNTPAAGAPTKPPGP&lt;sub&gt;124&lt;/sub&gt;</td>
<td>1466 (3402)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Expressed as pmol/min. Background values without exogenous substrate subtracted.

<sup>b</sup>Peptide substrate concentration; 0.25 mU of enzyme per reaction was used and the reaction time was 1.0 h.

![Fig. 5. MALDI-TOF mass spectra of synthetic wild-type and mutant gC-1 peptide glycosylated *in vitro* by GalNAcT-2 for 0 h, 1 h, and 24 h. The m/z values of peaks of differently glycosylated peptides are indicated. Calculated numbers of attached GalNAc residues are indicated within brackets.](https://academic.oup.com/glycob/article-abstract/14/7/571/643381)
cells; and (2) cell-to-cell spread of newly produced virus as manifested in differences in plaque morphology. This latter phenomenon involves mainly other glycoprotein-dependent processes than binding of free virus to cells, which is outlined more in detail later. Owing to the role of gC-1 in HSV-1 binding to cell surface heparan sulfate and chondroitin sulfate, an analysis of the attachment kinetics of mutant and rescue virus was performed (Figure 6). The target cells were GMK cells, mouse L cells, and two mutant cells of L cell origin, that is, cells synthesizing chondroitin sulfate but not heparan sulfate (Gro2C cells), and cells synthesizing heparan sulfate but not chondroitin sulfate (sog9 EXT-1 cells) (Gruenheid et al., 1993; McCormick et al., 1998).

We found that the adsorption graphs of HSV-1gC-1(114K, 117R)A or HSV-1Rescue to different types of cells. The attachment assay was performed by adding the indicated amounts of radiolabeled virus to monolayers of GMK AHI, Gro2C cells, sog9 EXT-1 cells, and L cells, respectively. All virus preparations used for experimentation were derived from HSV-1gC-1(114K, 117R)A- or HSV-1Rescue-infected GMK cells. Mean values and SEMs (n = 3) are indicated.

### Table II. Analysis of number of GalNAc residues incorporated and sites of occupancy with GalNAc-T2

<table>
<thead>
<tr>
<th>Acceptor peptide</th>
<th>Amino acid sequence</th>
<th>GalNAc incorporated</th>
<th>Acceptor sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>gC-1Rescue</td>
<td>106PKNNTPKGRTKPGP124</td>
<td>1/2</td>
<td>S115,T119</td>
</tr>
<tr>
<td>gC-1(114K, 117R)A</td>
<td>106PKNNTPAAPGTPKPGP124</td>
<td>2/3</td>
<td>T111,S113,T119</td>
</tr>
</tbody>
</table>

*Total number of moles of GalNAc incorporated after 1 h (figure before slash) or after 24 h (terminal reactions; figure after slash) as described under Materials and methods.

bAcceptor sites identified for human GalNAc-T2 in exhaustive/terminal reactions, with numbers indicating positions in the corresponding acceptor peptide. Identification of the acceptor sites were performed with Edman degradation.

**Fig. 6.** Binding of HSV-1gC-1(114K, 117R)A or HSV-1Rescue to different types of cells. The attachment assay was performed by adding the indicated amounts of radiolabeled virus to monolayers of GMK AHl, Gro2C cells, sog9 EXT-1 cells, and L cells, respectively. All virus preparations used for experimentation were derived from HSV-1gC-1(114K, 117R)A- or HSV-1Rescue-infected GMK cells. Mean values and SEMs (n = 3) are indicated.
HSV-1<sub>Rescue</sub> for the two highest virus concentrations. These results, which were reproducible in three complete, consecutive triplicate experiments (<i>p</i> < 0.005, <i>n</i> = 9), would indicate that the two basic amino acids at positions 114 and 117 are necessary for full binding capacity of HSV-1 to chondroitin sulfate but not to heparan sulfate. Attempts to use peptide attachment blocking experiments failed to assign any direct role of K<sup>114</sup> and R<sup>117</sup> in binding to cell surface glycosaminoglycans; only small inhibitory effects (20%) were achieved using a blocking peptide concentration of 50 μM and in fact the mutant peptide was a better inhibitor of wild type as well as mutant virus than the wild-type peptide (data not shown).

We also investigated whether the mutations induced affected the plaque morphology of infected GMK, L cells, and Gro2C cells (Figure 7), because this is a phenomenon largely related to late functions of HSV-1 glycoproteins in the infectious cycle (reviewed by Johnson and Huber, 2002; Rajcani and Vojvodova, 1998). We found that the plaques generated by HSV-1<sub>gC-1(114K,117R)</sub> were almost 50% smaller than those formed by HSV-1<sub>Rescue</sub> (<i>p</i> < 0.001). The difference in plaque size between HSV-1<sub>gC-1(114K,117R)</sub> and HSV-1<sub>Rescue</sub> was observed for GMK and L cells as well as Gro2C cells. This similarity between the three cell types was in contrast to the finding that the different attachment rate between mutant and wild-type virus was evident only for one of the investigated cells, Gro2C. This supports the notion that the changed plaque morphology on one hand and attachment rate to Gro2C on the other hand each are caused by different and independent consequences of the altered O-linked glycosylation signal. It is important that although the parental gC-1 null virus strain, designated gC-39, produced syncytial plaques (syncytial and nonsyncytial morphology depicted in Figure 8), we found that neither the plaques induced by HSV-1<sub>gC-1(114K,117R)</sub> nor by HSV-1<sub>Rescue</sub> were syncytial, suggesting that no alterations in the membrane fusion capacity of HSV-1 glycoprotein were involved in the plaque size transition between these two latter virus strains. As an extra control, we analyzed confluent plates with more than 200 plaques each of HSV-1<sub>gC-1(114K,117R)</sub> and HSV-1<sub>Rescue</sub> using a gC-specific monoclonal antibody. Interestingly, we found only a few nonstained gC-negative revertant plaques from plates infected with mutant as well as rescue virus, but as expected these plaques were of the same syncytial, large phenotype as the parent gC-1 negative virus strain. Altogether, the results indicated that the O-glycosylation peptide signal also affected viral spread in cell culture even in the absence of cell surface heparan sulfate.

Discussion

The findings of this article are of significance at two levels. First we have identified what seems to constitute labile elements in an O-linked glycosylation signal of the highly O-glycosylated protein gC-1. Thus, by changing two basic amino acids of the glycosylation signal that were not targets themselves for O-linked glycosylation, it was possible to generate a mutant gC-1 equipped with a considerably higher degree of O-linked glycosylation. In fact, the SDS–PAGE mobility shift suggested a difference of more than 8000 Da between mutant and wild-type gC-1, implying that the extra content of O-linked glycans of mutant gC-1 could be as much as 40 monosaccharide units. Although care should be taken when calculating absolute masses for the carbohydrate complement of a glycoprotein from electrophoretic data (Olofsson and Bolmstedt, 1998), our reported increase in glycosylation is based on SDS–PAGE results from continuous as well as gradient polyacrylamide gels, representing two different principles, sieving and stacking, respectively, for separation. Moreover, these systems have demonstrated a good correlation between the calculated carbohydrate content and electrophoretic mobility for...
mutant viral glycoproteins, lacking different numbers and combinations of N-glycosylation sites (Bolmstedt et al., 1991; Olofsson et al., 1999). Second, the peptide glycosylation signal harboring these two amino acids seems to influence two different processes in the HSV-1 life cycle: viral binding to cell surface chondroitin sulfate and viral cell to cell spread, at least as it is manifested in plaque morphology. Thus the present wild-type gC-1 O-linked glycosylation signal may be suboptimum for maximal O-linked glycosylation but optimum for maximal biological functionality.

The in vitro studies of O-linked glycosylation with individual GalNAc-transferases, using synthetic peptides as substrates, revealed some of the mechanistic details behind the increased O-linked glycosylation of gC(114K,117R)A. We choose GalNAc-T1, -T2, -T3, and -T6 because these are enzymes that function with naked peptide substrates and do not require prior GalNAc-glycosylation by other isoforms. They also represent the best characterized isoforms both in terms of specificity and expression patterns. GalNAc-T1 and -T2 further represent the most universally expressed isoforms (for a review, see Hassan et al., 2000a). Of the investigated GalNAc-transferases, only GalNAc-T2 demonstrated efficient activity with the wild-type and mutant peptide substrates, but the mutant peptide sequence was a significantly better substrate than the wild type. Furthermore, amino acid sequencing and MALDI-TOF MS showed that the mutant peptide was glycosylated with three moles of GalNAc (T111, S113, and T119), whereas the wild-type peptide was only glycosylated with two moles of GalNAc (T119 and S113). Thus the presence of positively charged amino acids among serine and threonine residues in the wild-type gC-1 peptide is likely to direct a lower density of O-glycosylation in this region, although the net difference was only one additional GalNAc residue attached with the mutant peptide.

It is clear that the total number of extra monosaccharides of O-linked glycans of gC-1(114K,117R)A compared with wild-type glycoprotein is considerably higher than what could be harbored in one extra O-linked glycan of gC-1(114K,117R)A. Considering that most of the O-linked glycans of gC-1, produced in GMK cells, constitute structures up to tetra- or possibly pentasaccharides (Lundström et al., 1987a), it appears unlikely that the contribution one or possibly two new O-linked glycans of gC-1(114K,117R)A, as suggested from the peptide model study, is sufficiently large to accommodate that many monosaccharides. Hence our results suggest that the induced mutations also affected the O-linked glycans of regions outside the peptide range (residues 106 and 124), most likely the domain delimited by amino acids 40 and 100, where numerous clustered O-linked glycans are harbored (Biller et al., 2000; Olofsson, 1992). This notion is in several ways in accordance with our current view of the temporal regulation of O-linked glycosylation. First, some GalNAc-transferase isoforms are not able to add GalNAc to a serine or threonine unless an adjacent serine or threonine residue is preglycosylated (Bennett et al., 1998). Thus it is possible that the facilitated addition of the first few GalNAc residues in the mutated gC-1 may pave the way for subsequently acting GalNAc-transferases, resulting in further increase in O-glycan density of gC-1 outside the peptide substrate sequence studied in this report. GalNAc-transferases further contain lectin domains with specificity for GalNAc-glycopeptides (Hassan et al., 2000b), and these are involved in enhancing the O-glycosylation density and possibly have other roles in directing O-glycosylation. Thus a seemingly marginal improvement of the O-glycosylation

Fig. 8. Morphology of nonsyncytial HSV-1 Rescue (A) and syncytial HSV-1 strain gC-39 plaques. Bar corresponds to 200 μ. To improve visualization of plaque fine structure, no immunostaining was applied.
signal could have profound effects on the total content of O-linked glycans in a glycoprotein, such as gC-1. The notion that gC-1(114K,117R)A produced in cells with an intact O-glycosylation machinery contains larger O-linked glycans reflects a general increase in the size of each O-linked glycan rather than an increased number of O-linked glycans is supported by the comparison by the studies of gC-1 Rescue and gC-1(114K,117R)A, produced in C1300 cells, containing only O-linked monosaccharides and to some extent sialylated GalNAc (STn) (Lundström et al., 1987b).

The induced modification of the O-glycosylation changed the biological properties of HSV-1 in two ways. First, the decrease in the ability of HSV-1 to bind to the surface of chondroitin sulfate–expressing cells, which represents the initial step in a series of interactions between HSV-1 and different cellular receptors (Campadelli-Fiume et al., 2000; Mårdberg et al., 2002). Second, the reduced size of the mutant virus plaques, which represents altered viral cell-to-cell spread, a more complex process as outlined in detail later. The chondroitin sulfate– and heparan sulfate–binding sites of gC-1 are largely overlapping, and several arginine residues and a few hydrophobic amino acid residues in the peptide stretch aa 129–160 are important determinants for binding to both types of glycosaminoglycan (Mårdberg et al., 2001, 2002; Trybala et al., 1994). Thus the increased content of O-linked glycans in gC-1(114K,117R)A may interfere either sterically or electrostatically with the interactions between gC-1 and chondroitin sulfate. Therefore one function of K114 and R117 could be to maintain an optimum net charge of a domain of significance for chondroitin sulfate binding, achieved by negative modulation of O-glycan sialylation, further supported by their cationic nature. Still, it is possible that identical domains of gC-1 are involved in chondroitin and heparan sulfate binding; the interference by O-linked glycans may affect the access for each type of glycosaminoglycan differently. Hence the dynamic responsiveness of this O-glycosylation signal may be a viral means to moderate the level of gC–chondroitin sulfate interactions.

Regarding the other biological effects of the modified O-linked glycosylation sequence, there is so far no specific role for gC-1 defined in the processes determining plaque size and viral cell-to-cell spread. The interplay between different HSV-1 gene products in promoting cell-to-cell spread is complex, involving several HSV-1 glycoproteins. Thus gD-1 seems to be a key factor for cell-to-cell spread of wild type HSV-1 strains in cultured cells (Cocchi et al., 2000), whereas cell-to-cell spread in keratinocytes may be mediated by gI/gE without any involvement of gD-1 (Huber et al., 2001; Johnson and Huber, 2002). Moreover, syncytial mutants of HSV-1 may spread among cells owing to the activity of gK-1, possibly supported by gC-1 (Pertel and Spear, 1996). If the function of gC-1 is regulatory, it may be difficult to assign a role to gC-1 in this multitude of interactions by use of deletion mutants. In spite of this, the present data suggest that the O-linked glycosylation signal of gC-1 constitutes one of several factors affecting the plaque size of HSV-1, probably by one or more of at least three conceivable ways. First, gC-1 interaction with cell surface chondroitin but not heparan sulfate is essential for the large plaque size phenotype of HSV-1. This explanation seems unlikely, because of the small decrease in chondroitin sulfate binding as observed for HSV-1gC-1(114K,117R)A compared with wild-type virus (Figure 4). Second, the O-linked glycans of gC-1 may modulate the cell-to-cell spread-promoting of other HSV-1 glycoproteins, including, gE/gI, gK, or gD. Third, the O-linked glycosylated peptide signal with its O-glycan array may itself be engaged in cell-to-cell spread or cell morphology activities affecting plaque size. This latter hypothesis is in line with both findings that (1) the degree of Ebola virus–induced cell detachment is proportional to the length of a used O-linked glycosylation signal in the Ebola virus glycoprotein (Simmons et al., 2002), and (2) the finding that such signals are engaged also on nonviral cell detachment mechanisms (Chervenak and Illsley, 2000).

Materials and methods

Cells and viruses

GMK-AHI cells were grown in Eagle’s minimum essential medium (EMEM) supplemented with 4% heat-inactivated fetal bovine serum and 0.05% Primacon RL substance. The murine fibroblast cell line LMTk-, clone ID, and its HSV-resistant variants gro2C, expressing chondroitin sulfate only (Gruenheid et al., 1993), and sog8 EXT-1 cells, expressing heparan sulfate but no other glycosaminoglycan marker (Mc Cormick et al., 1998), were grown in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum in 5% CO2 atmosphere. The mouse neuroblastoma cell line was propagated as previously described (Lundström et al., 1987b).

The mutated HSV strain HSV-1gC-1(114K,117R)A has two amino residue substitutions, specifically lysine and arginine residues at positions 114 and 117 replaced with alanine residues. The HSV-1 virus designated HSV-1Rescue was used as a wild-type control, and this virus expresses an unmodified gC gene introduced into the HSV-1 gC null strain gC-39 (Holland et al., 1984). HSV-1gC-1(114K,117R)A and HSV-1Rescue were originally constructed for studies on the heparin sulfate–binding site of gC-1, and both viruses have been characterized in detail previously (Mårdberg et al., 2001). To avoid possible differences in other genes than gC-1, the wild-type and the mutated gC-1 genes were inserted into gC-39 using identical protocols for homologous recombination to produce infectious virus clones. Three plaques each of mutant and wild-type virus were selected for further experimentation and the entire gene for gC-1 was control sequenced (Mårdberg et al., 2001). The gC-1-null strain gC-39 produced essentially syncytial plaques, whereas the plaques of HSV-1gC-1(114K,117R)A and HSV-1Rescue were nonsyncytial. The number of physical viral particles was calculated as previously described (Mårdberg et al., 2001). The virus particle/PUF ratio varied between 500–1000, with no significant difference between mutant and rescue virus. The term gC(K114, R117)A is used for designation of the mutated gC-1 protein.

In vitro assessment of O-glycosylation capacity

Polypeptide GalNAc-transferase assays were carried out essentially as described using soluble, secreted, purified
recombinant human GalNAc-T1, -T2, -T3, and -T6, expressed in insect cells (Bennett et al., 1996; Wandall et al., 1997). Acceptor peptides, representing rescue gC-l, K114, R117A, and gC(K114, R117)A, 10^6PKNNTTPAASGAPTKPPGP124, were synthesized by Chiron Mimotopes (Victoria, Australia), and purified by the manufacturer to 99% purity. Briefly, the assays were performed in 25 μl total reaction mixture containing 25 mM cacodylate (pH 7.4), 10 mM MnCl_2, 0.25% Triton X-100, 200 μM UDP-[^14C]-GalNAc (3,700 cpm/nmol) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and 250 μM UDP-GalNAc, 15 μg acceptor peptide for assays with GalNAc-T1, -T2 and -T3 and 0.78 to 25 μg acceptor peptide for assays with GalNAc-T6, and 0.25 μM of each purified GalNAc-transferase, respectively. Soluble, secreted forms of the human GalNAc-transferases analyzed were expressed in High Five cells, grown in cell-free media, and purified to near homogeneity with specific activities of 0.6 U/mg for GalNAc-T1, 0.5 U/mg for GalNAc-T2, 0.5 U/mg for GalNAc-T3, and 2.35 U/mg for GalNAc-T6 measured using peptides derived from MUC2, MUC1, and MUC7 human mucin tandem repeats as described previously (Wandall et al., 1997). Products were quantified by liquid scintillation counting after chromatography on Dowex-1, octadecyl silica cartridges (Mallinkrodt Baker Inc, Philipsburgh, NJ), or HPLC (PC3.2/3 or mRPC C2/C18 SC2.1/0 Pharmacia, Smart System; Amersham Pharmacia).

Evaluation of GalNAc incorporation into peptides was performed by MALDI-TOF mass spectrometry (Voyager DE; AME Bioscience, Toroed, Norway) as described previously (Schwientek et al., 2002). Sites of incorporation was determined by Edman degradation on an Applied Biosystems Procise HT 494 sequencer using glass fiber filters precycled with BioBrene Plus (Applied Biosystems, Foster City, CA) as immobilizing support. (N-terminal sequence analysis was carried out at the Protein Analysis Center, Kardinska Institute, Stockholm, Sweden.) The peptide samples were aliquots of HPLC fractions isolated in a mixture of acetonitrile, 0.1% trifluoroacetic acid and water. The samples were spotted onto the BioBrene Plus treated and precycled glass fiber filters. The PTH derivatives were separated on-line using a Brownlee Spheri-5PTH, 5 micron, 220 × 2.1 mm C18 column at 0.325 ml/min. N-terminal sequence analysis was carried out using a Procise HT instrument (Applied Biosystems) after application of samples in solution to precycled BioBrene-treated glass fiber filters (Applied Biosystems).

Metabolic labeling and immunoprecipitation of HSV-1 glycoproteins

Confluent layers of GMK cells in 6-well culture plates were washed two times with serum-free EMEM and infected with HSV-1_rescue or HSV-1_gC-l(114K,117R)A at a m.o.i. of 3. The virus was allowed to adsorb to the cells for 1 h at 37°C, followed by addition of EMEM, containing antibiotics. After incubation in CO_2-incubator for 4 hours 50 μCi/ml D-[6-^3H]-glucosamine hydrochloride ([^3H]-GlcN; Amersham Pharmacia, 30 μCi/mmol) was added. The cells were radio-labelled for 20 hours until harvest. The labeled culture supernatant was removed from cells and supplemented with 1% (v/v) NP40 and 1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl, HCl:p-aminoethylbenzenesulfonyl fluoride, HCl; Calbiochem/Novabiochem, San Diego CA). Cells were detached using rubber policemen, washed twice with TBS (tris-buffered saline; 150 mM NaCl, 50 mM tris HCL, pH 7.5) and resuspended in 1 ml TBS containing, 1% (v/v) NP40 and 1 mM AEBSF. Cell suspensions were sonicated on ice followed by centrifugation to remove cell debris. Radioimmunoprecipitation was carried out essentially as previously described (Lundström et al., 1987a). Heat-inactivated formalin-fixed S. aureus, coated with anti-mouse antibodies (Dakopatts, Glostrup, Denmark), were mixed with anti-HSV-1gC-specific mouse monoclonal antibodies B1C1 or C4H11B for 2 hours at 4°C (Olofsson et al., 1983; Sjöblom et al., 1992). The antibody-staphylocococcus complex was then washed three times in TTB [TBS, containing 1% Triton X-100 and 0.1% bovine serum albumin (BSA)] and mixed with labeled culture supernatants or labeled cell lysates overnight at 4°C. The samples were washed twice in TTB and twice in TBS prior to analysis by homogenous SDS-PAGE (9.25% polyacrylamide gels) or gradient gel SDS-PAGE (NuPAGE 4-12%; Invitrogen; Carlsbad, CA) and fluorography (Amersham Pharmacia). In some experiments gC-1 was detected by immunoblot, using a monoclonal antibody specific for a linear epitope (Sjöblom et al., 1992).

In some experiments radiolabeled gC-1 was affinity-purified as previously described and subjected to sialidase (V. cholerae, Behringwerke, Marburg, Germany; 100 U/ml) treatment at pH 4.5 for 2 h at 37°C. The glycoprotein and the released sialic acid were separated by gel filtration on short disposable Sephadex G-25 columns (Amersham Pharmacia).

N-Glycosidase (N-glycanase) digestion of immunoprecipitated glycoproteins

The enzymatic elimination of N-linked glycans was performed essentially as previously described (Olofsson et al., 1999). Briefly, [^3H]-GlcN-labeled culture supernatants were immunoprecipitated as described above but after final wash the samples were resuspended in 10 μl 20 mM sodium phosphate buffer pH 7.5, containing 10 mM EDTA, 0.005% sodium azide, 1% (v/v) β-mercaptoethanol, 1% (w/v) sodium dodecyl sulphonate (SDS) and incubated for 20 minutes at 37°C. The glycoprotein was denatured and eluted from the Staphylococci by boiling for 5 minutes followed by centrifugation. The samples were then diluted 10 times in 20 mM sodium phosphate buffer pH 7.5 containing, 10 mM EDTA, 10 mM sodium azide, 1% (v/v) β-mercaptoethanol, 2% (v/v) NP40 to avoid SDS denaturation of PnGase. PnGase (6 μl of 2001U/ml; Roche Applied Sciences, Stockholm) was added to samples prior to incubation at 37°C for 20 hours. After acetone precipitation samples were finally analyzed on SDS-PAGE and autoradiography (Amersham Pharmacia).

Purification of extracellular virus

The purification of radiolabeled HSV-1 rescue and mutant strains were performed as previously described (Mårdberg et al., 2001). In brief, roller bottle cultures of GMK-AH1
cells were infected with either virus strain. Following virus adsorption the cells were washed, and 45 ml fresh EMEM supplemented with 40 μCi/ml of [methyl-3H]-thymidine (25 Ci per mmol, Amersham) was added. The cells were incubated for further 48 h, and thereafter the virus was pelleted from culture medium and purified using a tree-step discontinuous gradient of sucrose as described (Karger et al., 1995). Unless otherwise stated, purified virus was resuspended in PBS containing 0.1% BSA and stored at –70°C. The numbers of virus particles in the purified preparations were calculated based on the determination of the DNA content (Karger et al., 1995).

**Binding of radiolabeled virus to cells**

Monolayers of GMK-AHI, Gro2C cells, sog9 EXT-1 cells and L cells in 96-well plates were precooled for 30 min at 4°C, then washed twice with cold PBS and blocked for 1 h at 4°C with PBS-BSA. Different virus strains, adjusted to contain the same initial number of viral particles, were serially diluted in PBS-BSA, as indicated in the figure legends. Equal 50-μl portions of each virus dilution were added in triplicate, and the plates were left for virus adsorption for 5 h at 4°C under continuous agitation. Subsequently, the cells were washed three times in cold PBS to eliminate unadsorbed virions and lysed in 5% SDS. The radioactivity was determined by scintillation counting, and the results were expressed as the percentage of attached virions relative to the number of virus particles originally added to the cells.

**Virus plaque size assay on glycosaminoglycan-deficient cells**

Monolayers of GMK-AHI and L cells in 6 well plates were infected with about 200 pfu per strain of the mutated HSV, gC(114K,117R)A, as well as the rescue strain (HSV-1_rescue). The monolayers were washed in complete medium, DMEM high glucose (L cells) and EMEM (GMK AHI) supplemented with penicillin and streptomycin prior to virus addition. The virions were allowed to adsorb at 37°C for 2 h, and thereafter the cells were washed three times in complete medium, and 1% methylcellulose solution was added. The cells were incubated for 3 or 4 days, as indicated in figure legends, and nonconfluent plaques were detected by black plaque assay with the gC-1-specific MAB B1C1 (Nilheden et al., 1983; Olofsson et al., 1983). The plaque size was determined essentially as described by Baigent et al. (2001). In brief, the plates were photographed together with a ruler, using a computer-assisted CCD camera, and the largest diameter of each plaque was determined at 3× magnification, using the ruler scale for standardization. A mean value was calculated for each strain and cell line, and the statistical significance of the measurements was determined by using Student t-test.

**Acknowledgments**

This work was supported by grants from the Swedish Medical Research Council (Grants 9083, 9483, and 11225); the Medical Faculty, University of Göteborg; and the Danish Research Council. The skillful technical assistance of Richard Lymer is gratefully acknowledged.

**Abbreviations**

DMEM, Dulbecco’s modified Eagle’s essential medium; EMEM, Eagle’s minimal essential medium; GMK, green monkey kidney; HSV-1 herpes simplex virus type 1; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

**References**


Cocchi, F., Menotti, L., Dubreuil, P., Lopez, M., and Campadelli-Fiume, G. (2000) Cell-to-cell spread of wild-type herpes simplex virus type 1, but not of synctial strains, is mediated by the immunoglobulin-like receptors that mediate virion entry, nectin1 (PRR1/ HSV-1 binding protein) and nectin2 [PRR2(Htr2)]. *J. Virol.*, 74, 3909–3917.


Friedman, H.M., Glorioso, J.C., Cohen, G.H., Hastings, J.C., Harris, S.L., and Eisenberg, R.J. (1986) Binding of complement component C3b to glycoprotein gC of herpes simplex virus type 1: mapping of gC-binding sites and demonstration of conserved C3b binding in low-passage clinical isolates. *J. Virol.*, 60, 470–475.


Holland, T.C., Homa, F.L., Marlin, S.D., Levine, M., and Glorioso, J.
Markine-Goriaynoff, N., Georgin, J.P., Goltz, M., Zimmermann, W.,
Characterization of glycoprotein C-negative mutants of herpes simplex
for infection by pseudorabies virus.
Mettenleiter, T.C. (1995) Cell surface proteoglycans are not essential
195±207.
Johnson, D.C. (2001) Herpes simplex virus with highly reduced gD
J. Virol.
exhibit multiple phenotypes, including secretion of truncated glyco-
O-linked oligosaccharides.
II. Demonstration of cell-specific galactosyltransferase essential
for formation of O-linked oligosaccharides.
Virology, 161, 195±207.
Virology, 161, 385±394.
Mårdberg, K., Trybala, E., Glorioso, J.C., and Bergstrom, T. (2001)
Mutational analysis of the major heparan sulfate-binding domain of
herpes simplex virus type 1 glycoprotein C. J. Gen. Virol., 82, 1941±
1950.
glycoprotein C is necessary for efficient infection of chondroitin sulfate-
Markine-Goriaynoff, N., Georgin, J.P., Goltz, M., Zimmermann, W.,
saminyltransferase-mucin encoded by bovine herpesvirus 4 was acquired from an ancestor of the African buffalo. J. Virol., 77, 1784±1792.
McCormick, C., Leduc, Y., Martindale, D., Mattison, K., Esford, L.E.,
Olofsson, S., Biller, M., Bolmstedt, A., Mårdberg, K., Leckner, J.,
herpes simplex virus type 1 envelope glycoprotein gC. Glycobiology, 9, 73±81.
Olofsson, S., Sjöblom, I., Lundström, M., Jeansson, S., and Lycke, E.
Rux, A.H., Moore, W.T., Lambris, J.D., Abrams, W.R., Peng, C.,
Friedman, H.M., Cohen, G.H., and Eisenberg, R.J. (1996) Disulfide
bond structure determination and biochemical analysis of glyco-
protein C from herpes simplex virus. J. Virol., 70, 5455±5465.
Schwientek, T., Bennett, E.P., Flores, C., Thacker, J., Hollmann, M.,
Reis, C.A., Behrens, J., Mandel, U., Keck, B., Schäfer, M.A., and
UDP-N-acetylgalactosamine:polypeptide N-acetylgalactosaminyl-
transferases in Drosophila, Caenorhabditis elegans, and mammals. One
subfamily composed of I(2)35Aa is essential in Drosophila. J. Biol.
Chem., 277, 22623±22638.
Serafini-Cessi, F., Dall’Olio, F., Pereira, L., and Campadelli-Fiume, G.
Simmons, G., Wool-Lewis, R.J., Baribaud, F., Netter, R.C., and
Antigenic structure of the herpes simplex virus type 1 glycoprotein C: demonstration of a linear epitope, situated in an environment of highly conformation-dependent epitopes. Amnis, 100, 229±236.
Stannard, L.M., Fuller, A.O., and Spear, P.G. (1987) Herpes simplex virus glycoproteins: association with different morphological entities project-
Trybala, E., Bergström, T., Svennerholm, B., Jeansson, S., Glorioso, J.C.,
gC-1 involved in binding to cell surface heparan sulfate. J. Gen. Virol., 75, 743±752.
Wandall, H.H., Hassan, H., Mirgorodskaya, E., Kristensen, A.K.,
Roepstorff, P., Bennett, E.P., Nielsen, P.A., Hollingsworth, M.A.,
Substrate specificities of three members of the human UDP-N-
acetylated-galactosamine:polypeptide N-acetylgalactosaminyl-
transferase family, GalNAc-T1, -T2, and -T3. J. Biol. Chem., 272, 23503±23514.
Vanderplaschen, A., Markine-Goriaynoff, N., Lomonte, P., Suzuki, M.,
glucosaminyl-transferase is encoded by bovine herpesvirus type 4.