Herpes simplex virus type 1 glycoprotein C is necessary for efficient infection of chondroitin sulfate-expressing gro2C cells

Kristina Mårdberg,1 Edward Trybala,1 Frank Tufaro2 and Tomas Bergström1

1 Department of Clinical Virology, Göteborg University, Guldhedsgatan 10B, S-413 46 Göteborg, Sweden
2 Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Boulevard, Vancouver, BC, Canada V6T 1Z3

The role of glycoprotein C (gC) for binding of herpes simplex virus type 1 (HSV-1) to cell surface chondroitin sulfate (CS) and the consequences of this interaction for virus attachment and infectivity were studied. To this end, a panel of HSV-1 gC mutants, including a gC-negative (gC−) variant, and mouse fibroblasts expressing either cell surface CS or heparan sulfate (HS) were used. Comparing gC-positive (gC+) and gC− viruses in terms of their attachment to and infection of CS-expressing cells indicated that gC was essential for both functions. Furthermore, purified gC bound efficiently to isolated CS chains. However, hypertonic NaCl disrupted this interaction more easily as compared to the binding of gC to HS. Also, native and selectively desulfated heparins were approximately 10 times more efficient at inhibiting gC binding to CS-expressing cells than binding to HS-expressing cells. Experiments with the HSV-1 gC mutants revealed that specific, positively charged and hydrophobic amino acids within the N-terminal part of the protein were responsible for efficient binding as well as infectivity in both CS- and HS-expressing cells. When the infectivity of the gC mutants in the two cell types was compared, it appeared that more residues contributed to the infection of CS-expressing cells than to infection of HS-expressing cells. Taken together, analysis of gC function in cell systems with limited expression of glycosaminoglycans revealed that gC could interact with either CS or HS and that these interactions exhibited subtle but definite differences as regards to the involved structural features of gC, ionic strength dependency as well as sensitivity to specifically desulfated heparin compounds.

Introduction

Herpes simplex virus type 1 (HSV-1) entry of cells is a cascade of events that is initiated by virus attachment to glycosaminoglycan (GAG) molecules in the form of heparan sulfate (HS) (WuDunn & Spear, 1989). This and later reports have shown that human herpesviruses representing all subfamilies, including the alphaferpesviruses HSV-2 (WuDunn & Spear, 1989) and varicella-zoster virus (Zhu et al., 1995), the betaferpesviruses human cytomegalovirus (Kari & Gehrz, 1992) and human herpesvirus type 7 (HHV-7) (Secchiero et al., 1997) and the gammaherpesviruses HHV-8 (Akula et al., 2001) utilize HS as an initial receptor.

In HSV-1, binding to cell surface HS during attachment is mediated primarily by glycoprotein C (gC), in which a functional domain responsible for this interaction has been defined (Herold et al., 1991; Mårdberg et al., 2001; Svennerholm et al., 1991; Tal-Singer et al., 1995; Trybala et al., 1994). Under some conditions, HSV-1 may utilize chondroitin sulfate (CS) for attachment to the cell surface (Banfield et al., 1995b), but the HSV glycoprotein responsible for CS binding has not been identified. In addition, it has been reported that CS may serve as a receptor for some other viruses (Bruett et al., 2000; Hsiao et al., 1999). The CS molecule is, in parallel with HS, a GAG that is found in many tissues in the human body, including the epidermis and connecting basal membrane of the human skin, where HSV-1 causes tissue damage during symptomatic reactivation (Huff et al., 1981; Murdoch et al., 1994; Sorrell et al., 1999; Zimmermann et al., 1994). In polarized MDCK cells, which express CS predominately on their apical surfaces (Kolset et al., 1999), HSV-1 infection by this route was shown to be gC-dependent (Sears et al., 1991). These two families of GAG molecules show several similar
features, such as the formation of long saccharide chains that are modified extensively by sulfation at selected positions and exposure into the extracellular matrix. It is therefore of interest to investigate features of HSV-1 interaction with HS and CS under comparable conditions.

Using HSV-1, mutant gro2C cells exposing only CS on the cell surface and variants deficient in HS and CS expression were sequentially derived from mouse fibroblast L cells, as reported earlier (Banfield et al., 1995a; Gruenheid et al., 1993). In a virus-resistant cell line lacking both CS and HS expression (sog9 cells), permissiveness to HSV-1 infection was restored by transfection of the EXT-1 gene from HeLa cells, enabling a regained capacity to synthesize HS (McCormick et al., 1998). This gave us the opportunity to compare the role of gC and its functional domain during virus entry in cells sharing the same ancestry but expressing both HS and CS, either HS or CS or lacking the sulfated GAG molecules altogether. Using a panel of HSV-1 strains carrying point-mutations in gC, a gC-positive lacking the sulfated GAG molecules altogether. Using a panel of HSV-1 strains carrying point-mutations in gC, a gC-positive lacking the sulfated GAG molecules altogether. Using a panel of HSV-1 strains carrying point-mutations in gC, a gC-positive lacking the sulfated GAG molecules altogether. Using a panel of HSV-1 strains carrying point-mutations in gC, a gC-positive lacking the sulfated GAG molecules altogether.
HSV-1 glycoprotein C and chondroitin sulfate desulfated heparin fragments (a gift from D. Spillmann, Uppsala, Sweden). Then, the heparin–gC mixtures were left to adsorb to monolayers of L, gro2C and sog9 EXT-1 cells for 1 h at 4 °C, washed three times and the amount of bound gC was detected by ELISA after fixation of the monolayers with 0.25% glutaraldehyde.

Assessment of infectivity in GAG-deficient cells. The HSV gC mutant strains, as well as KOS321 and gC−39, were titrated on GMK-AH1 cells and approximately 300 p.f.u. per strain was added in duplicate to monolayers of GMK-AH1, L, gro2C, sog9 and sog9 EXT-1 cells. The monolayers had been washed in DMEM medium before the virus was added. Virions were allowed to adsorb for 2 h at 37 °C. The cells were then washed three times in DMEM and 1% methylcellulose solution was added. The cells were incubated for 4 days and stained with crystal violet. The number of virus plaques was expressed as a percentage of plaques formed on GMK-AH1 cells.

Results

Treatment of GAG-degrading enzymes impairs HSV-1 attachment to L cell variants deficient in GAG expression

Although it has been shown that cell surface CS can act as an initial receptor for HSV-1 infection in the absence of HS, the glycoprotein responsible for this interaction is not defined clearly (Banfield et al., 1995b). To investigate the role of gC in CS binding, we utilized the following cell lines: mouse L fibroblasts expressing both CS and HS, the L cell-derived gro2C cells exposing only CS (Gruenheid et al., 1993) and, as negative control, sog9 cells expressing neither HS nor CS (Banfield et al., 1995b). The results in Fig. 1 show attachment of the gC+ strain KOS321 and the gC− strain gC−39 to L, gro2C and sog9 cells and the effect of pre-treatment of these cells with GAG lyases on virus binding.

In untreated cells, the gC+ virus attached to a lesser extent to gro2C cells in comparison with L cells, which is in accordance with previous results (Banfield et al., 1995a). In gro2C cells, as well as in L cells, the gC− virus showed deficient attachment as compared to the gC+ virus. In sog9 cells, neither of these viruses bound at notable levels. After chondroitinase treatment, the superior attachment of the gC+ virus in comparison to the gC− virus was abolished in gro2C cells (Fig. 1b), but not in L cells, indicating that CS alone was responsible for gC-mediated attachment in the former cell line. Furthermore, a role for CS in attachment was also evident in heparinase-treated L cells when the attachment of the gC+ virus was assayed and compared to binding to the same cells treated with both heparinase and chondroitinase (Fig. 1a). Heparinase treatment alone reduced binding by a third, while removal of both HS and CS reduced attachment of the gC+ virus to the level of the gC− virus variant. The fact that similar enzyme treatments had no or slight effect on the attachment of the gC− virus to the same cell line, again indicated that gC was responsible for interaction with CS during the attachment step. When sog9 cells were treated with HS or CS lyases, either alone or in combination, no additional reduction in virus attachment was observed (Fig. 1c), confirming that these cells did not express any GAG molecules that were of importance for virus binding. Taken together, these findings indicate that gC mediated virus binding to cell surface CS in the absence of HS.
Fig. 2. Binding of purified gC to CS and HS isolated from L, gro2C and sog9 EXT-1 cells. Subconfluent cell monolayers were labelled with \(^{35}\)S and subjected to protease treatment, whereafter the GAG molecules were bound to a DEAE-Sepharose column and eluted with 2 M NaCl. To obtain HS from L cells, CS was removed by chondroitinase ABC treatment. Preparations of HS or CS were incubated with serial fivefold dilutions of purified gC in PBS supplemented with BSA. A portion of the CS derived from gro2C cells was degraded with chondroitinase ABC and also tested for gC binding. Bound GAG molecules were trapped on nitrocellulose filters and their amounts were determined by liquid scintillation counting. Results are shown as the mean ± SE of three separate experiments.

**Purified gC binds CS isolated from gro2C cells**

Results shown in Fig. 1 suggested that gC mediated HSV-1 attachment to cell surface CS on gro2C cells. However, these data do not provide any evidence that gC alone can form a stable complex with CS, since other cellular or viral proteins might have contributed to the virus–cell interactions. Therefore, a direct binding assay of purified gC and CS was performed. CS isolated from gro2C cells, or HS isolated from L or sog9 EXT-1 cells, was mixed with immunoaffinity purified gC and the GAG–gC complexes formed were trapped on nitrocellulose filters. As shown in Fig. 2, purified gC bound to CS as well as to HS. To ensure the specificity of the CS–gC interaction, a subfraction of the CS preparation was pre-treated with chondroitinase ABC before mixing it with gC. The results (also shown in Fig. 2) showed that this treatment completely abolished the ability of CS to interact with gC. However, gC bound higher amounts of HS from L cells and similar amounts of the same molecule from sog9 EXT-1 cells as compared to CS from gro2C cells. This finding suggests that, even in cells from the same origin, quantitative and/or qualitative differences between HS chains may exist and influence the gC–HS interaction. Alternatively, the differences could be explained by the varying efficiency of GAG chain-labelling due to differences in GAG biosynthesis between the two cell lines.

The dependence on ionic strength of HS (from L and sog9 EXT-1 cells) or CS (from gro2C cells) in binding to gC was compared by mixing gC with either radiolabelled GAG in the presence of increasing NaCl concentration. The gC–CS interaction was weaker, i.e. more sensitive to increased NaCl concentrations, than that of gC and HS isolated from either source (data not shown). These results suggest that, although gC bound to gro2C-specific CS, this interaction was relatively more dependent on electrostatic forces than that of gC and L- or sog9 EXT-1-specific HS.

**Binding of gC to cell surface CS is inhibited by selectively desulfated heparins**

The finding that gC bound CS in solution prompted attempts to evaluate binding of the protein to the surface of
CS-expressing gro2C cells. Purified gC bound to all three cell types expressing surface GAG molecules. It was demonstrated earlier that binding of the gC+ virus to gro2C cells was more sensitive to inhibition by HS as compared to L cells (Banfield et al., 1995b). To further characterize differences between gC–HS and gC–CS interactions, we inhibited gC binding to the cell surfaces of L, gro2C and sog9 EXT-1 cells by heparin as well as selectively desulfated heparin (Fig. 3). Binding of gC to CS-expressing gro2C cells was more sensitive (approximately 10 times) to inhibition by heparin as well as by the selectively desulfated heparin, as compared to the HS-exposing L and sog9 EXT-1 cells (Fig. 3, compare b to a and c). In addition, N-desulfated heparin was equally efficient as an inhibitor as heparin on gro2C cells as well as on sog9 EXT-1 cells, a finding that implies that N-linked sulfates on this molecule contribute little to gC binding (Fig. 3, b and c). Although the lack of N-sulfate contribution to inhibition of CS binding of gC is explained by the absence of such sulfates on the CS chains, the experiments demonstrate a similarity in this property between the two cell lines expressing either HS or CS. No difference in inhibition by 2-O- and 6-O-desulfated heparin in gro2C cells was found.

**Specific amino acid residues of HSV-1 gC are involved in virus binding to CS**

A HS-binding domain (HSBD) has been identified on the N-terminal part of gC (Mårdberg et al., 2001; Trybala et al., 1994). Residues 129–160 delimited this domain, involving a motif of positively charged and hydrophobic residues. To investigate whether the HSBD of gC was also involved in binding to cell surface CS, we performed a direct attachment assay using a panel of HSV-1 gC mutants and the L cell-derived GAG-deficient cell lines. The results are shown in Fig. 4. The panel of virus mutants exhibited specific binding patterns on L (Fig. 4a), gro2C (Fig. 4b) and sog9 EXT-1 (Fig. 4d) cells, but not on sog9 cells (Fig. 4c). Virus strains carrying mutations within the residues 143–160 were impaired in all three GAG-exposing
Fig. 5. Infectivity of KOS321 and gC−39 viruses on L, gro2C, sog9 and sog9 EXT-1 cells. Approximately 300 p.f.u. (as determined by plaque titration on GMK-AH1 cells) of either virus strain was added per well in duplicate to all cell types (6-well plates). After 2 h of adsorption, monolayers were washed and 1% methylcellulose was added. After 4 days, the cells were stained and the virus plaques were counted. Results are expressed as a percentage of the control, i.e. the number of plaques formed on GMK-AH1 cells. Results are shown as the mean±SE of seven determinations.

Fig. 6. Infectivity of the gC mutants on L (a), gro2C (b), sog9 (c) and sog9 EXT-1 (d) cells. The assay was performed as in the legend to Fig. 5. Results are shown as the mean±SE of seven determinations.

cell lines, suggesting that the core of the HSBD of gC is essential for interactions with either CS or HS or with a combination of the two molecules exposed on L cells. Although the pattern of virus mutants reduced in binding to CS- and HS-expressing cells was similar, certain differences could be observed. In particular, some hydrophobic residues
were found to be more important for binding to gro2C cells than to L and sog9 EXT-1 cells. The virus mutants with alterations in either I142 or F146 were severely impaired in attachment to gro2C cells, but only slightly reduced in binding to L and sog9 EXT-1 cells. Finally, the poor binding and absence of differences between the mutants in attachment to sog9 cells indicated that the demonstrated loss of binding of some mutants to GAG-expressing cells was due to virus interaction with cell surface CS and/or HS.

An intact N-terminal part of gC is important for efficient virus infection of gro2C cells

To determine whether gC was also required for efficient infection of CS-expressing cells, the gC+ (KOS321) and gC− (gC−39) virus strains were tested for their infectivity in L, gro2C, sog9 and sog9 EXT-1 cells (Fig. 5). The panel of gC mutant strains, used in the above attachment assays, was also included to characterize further the role of particular amino acid residues in gC for infection of CS-expressing gro2C cells compared to HS-expressing L and sog9 EXT-1 cells (Fig. 6). To ensure equal amounts of infecting virus strains, all viruses were titrated on GMK-AH1 cells prior to assay, with the rationale that gC does not play a major part in attachment or infection of this cell line (Trybala et al., 1994).

In accordance with previous results, infectivity of KOS321 was reduced in CS-expressing gro2C cells and profoundly impaired in sog9 cells, where infectivity was rescued efficiently by the EXT-1 gene product to levels comparable with the parental L cells. As deduced from comparisons of infectivity between the gC+ and the gC− viruses, gC provided a profound advantage for infection of gro2C cells, but not for L cells expressing both HS and CS (Fig. 5). In addition, the low level of infectivity displayed by the gC− virus on gro2C cells (comparable to that seen in sog9 cells) indicated that other envelope glycoproteins were not able to rescue infectivity, as was seen in L cells and, to some degree, in sog9 EXT-1 cells. In parallel with the results of the attachment studies, residues situated within the HSBD were found to be important for infectivity in all of the GAG-expressing cell lines (Fig. 6). The most pronounced decrement in infectivity on L, gro2C and sog9 EXT-1 cells was observed in mutants with alterations within residues 143–160 and the T → F substitution at position 146 affected virus infectivity most severely in all three cell lines. In gro2C cells, this mutant failed to produce any plaques with the inocula used.

Some mutants, such as I142T and K(114,117)A were slightly more impaired in infectivity on gro2C cells than on L and sog9 EXT-1 cells, suggesting a greater importance of some positively charged as well as hydrophobic amino residues for infectivity in this cell line. However, such differences might be revealed more easily in gro2C cells where infectivity was more gC-dependent. In conclusion, gC facilitated infectivity in cells expressing either CS or HS and the functional HSBD of gC was responsible for this facilitation also in gro2C cells, most likely due to an ability of the domain to accommodate CS features expressed in gro2C cells.

Discussion

Herpesviruses, as do many enveloped viruses, commonly utilize GAG molecules as initial receptors. Although the role for such interactions during in vivo infection is currently unknown, it is well documented that the binding of HSV-1 to HS exposed on the surface of cultured cells initiates and enhances virus entry (Herold et al., 1991; WuDunn & Spear, 1989). However, in the absence of HS, HSV-1 may utilize CS as a receptor for attachment, as was demonstrated by experiments using HS-deficient gro2C cells (Banfield et al., 1995b). Here we show, using the same cell line, that the binding of HSV-1 to CS is mediated by gC, since a gC− virus was strongly impaired in attachment to gro2C cells. Moreover, this protein formed a chondroitin lyase-sensitive complex with CS molecules isolated from HS-deficient gro2C cells. By analysing the binding of a library of gC mutant viruses found previously to be defective in HS binding, we conclude that overlapping, but not identical, domains of gC are responsible for both HS and CS binding.

CS, a family of GAG molecules expressed ubiquitously in a plethora of human tissues (Bode-Lesniewska et al., 1996; Sorrell et al., 1999; Zimmermann et al., 1994), plays a significant role for several biological functions in vivo. In the nervous system, CS is involved in regulation of cell migration and axonal outgrowth (Bovolenta & Fernaud-Espinosa, 2000). Furthermore, CS is a major component of the human skin in, for example, the basal membrane layer of the epidermis (Bode-Lesniewska et al., 1996; Sorrell et al., 1999). The major subtypes of CS (A, B and C) all bear similarities to HS in two ways: the repeating disaccharide units consist of amino sugars and hexuronic acids and these units show a variable degree of sulfation along the GAG chains. Due to the vast complexity of synthesis and modification of HS as well as CS chains and methodological limitations in sequencing sugar chains, specific domains functioning as initial receptors for viruses and other microbes have hitherto been difficult to delineate.

To define a role for gC during virus entry, previous attempts have utilized successfully the apical route of infection of polarized MDCK cells (Sears et al., 1991). This cell line was demonstrated recently to expose CS preferably on the apical surface in contrast to the basolateral areas, which mainly expose HS (Kolset et al., 1999). Of the cells utilized in the present study, the HS-deficient cell lines gro2C and sog9 cells were sequentially isolated from mouse fibroblast L cells by selecting cell clones that were resistant to HSV-1 infection (Banfield et al., 1995a; Gruenheid et al., 1993). Here enzymatic removal of GAG molecules confirmed earlier results demonstrating the necessity of such molecules for virus entry in both L and CS-exposing gro2C cells and that infectivity of
sog9 cells was unaffected by this treatment (Banfield et al., 1995b; Laquerre et al., 1998; Tal-Singer et al., 1995). It is interesting to note that heparinase treatment alone did not profoundly affect virus attachment to HS/CS-expressing L cells, but that dual treatment, including chondroitinase, strongly reduced virus binding. Furthermore, in parallel with what was demonstrated earlier on polarized cells (Sears et al., 1991), HSV-1 was dependent on a functional gC for attachment to as well as infection of the CS-expressing gro2C cells. It should be noted that in a previous study, using another gC− virus construct in a related experimental setting, no difference between the gC+ and the gC− viruses in attachment to gro2C cells was found (Banfield et al., 1995b). Further studies comparing the gC− strains and rescued variants thereof might be needed to resolve the question.

HSV-1 infectivity in sog9 cells was found earlier to be rescued by transfection of these cells with the EXT-1 gene, an HS polymerase (Lind et al., 1998; McCormick et al., 1998). This gave us the opportunity to compare a cell line expressing CS (gro2C cells) with cells expressing HS but no other GAG molecules (sog9 EXT-1 cells). In the attachment assay, binding of the gC+ viruses (KOS321 and gC rescue) were grossly similar in the two cell lines, suggesting that CS was at least as competent a molecule for initial binding of gC as was HS. This finding was supported by the comparable results from binding of gC to isolated CS and HS from these two cell lines. Furthermore, as judged by the results of a library of gC mutants, overlapping, but not identical, domains of gC were utilized for attachment to the CS- and HS-expressing cells, respectively. For both HS and CS interactions, a functional domain of gC consisting of positively charged arginine residues interspersed with hydrophobic amino acids was essential. In addition to the importance of I142 for HS binding reported previously (Mårdberg et al., 2001), the T→F substitution at position 146 was found to affect virus attachment and infectivity severely in both gro2C and sog9 EXT-1 cells. Thus, hydrophobic interactions seem, in addition to electrostatic forces, to be decisive for CS as well as HS binding to gC.

When interactions between purified gC and GAG molecules and inhibitions thereof were studied, differences between CS and HS binding to gC were found. Our data evaluating the effect of increasing NaCl concentrations on gC complexes formed with HS or CS, where the gC−CS complex was more sensitive to an increasing NaCl concentration than the gC−HS complex, suggested a difference in the contribution of electrostatic forces between gC−HS and gC−CS interactions. This was in line with earlier data (Banfield et al., 1995b), indicating that HSV-1 virions bound more weakly to gro2C cells compared to the HS-expressing L cells. Furthermore, inhibition by heparin and desulfated derivatives showed a much stronger effect on binding of purified gC to the CS-expressing cells as compared to HS-expressing cells. These results suggest that gC displays different binding affinities versus HS and CS, a feature demonstrated earlier for another GAG-binding protein, HCII (Tollefsen, 1994).

The ability of gC to interact with both HS and CS could be understood if HS and CS were shown to share some structural features, such as similar gC-binding motifs. A prerequisite for such a comparison would be the definition of gC-binding sequences of HS and CS, respectively. In a previous work, we have shown that the gC−HS interaction was sulfate-dependent and that a minimal requirement was a heparin dodecamer containing at least one 2-O- and one 6-O-sulfate, possibly localized to the same disaccharide unit (Feyzi et al., 1997). A similar dependence on disulfated disaccharides might also be important for protein−CS interactions, as suggested by a recent report on platelet factor 4 interactions with this family of GAG molecules (Petersen et al., 1999). Although the CS molecules present on gro2C cells remain to be characterized, our preliminary results by removal of cell surface CS by specific chondroitinases suggested that structural features, which are removed by chondroitinase AC, but not B or C, were essential for infectivity of the gC+ KOS321 strain (unpublished observation). However, development of sequencing methods of CS chains, as has been achieved for HS, will be necessary to answer the question of whether HS and CS expose similar gC-binding motifs.

When analysing results of virus attachment as well as infectivity on L cells exposing both HS and CS chains, one striking feature was noticed: the gC-dependency that was obvious on the two cell lines exposing either of the GAG molecules was no longer discernible. Although we cannot explain this phenomenon at the present level of understanding, we suggest that the complexity introduced by the presence of dual receptors (HS and CS) with different gC affinities offers a greater possibility for other HS-binding viral proteins, such as gB, to display functional redundancy. Furthermore, the fact that expression and composition of CS and HS molecules in cell models utilized for studies of gC function in attachment and infectivity was, hitherto, given little attention might be of relevance for the contradictory results presented using similar experimental systems (Griffiths et al., 1998; Sears et al., 1991).

The significance of the gC−CS interaction demonstrated here for in vivo infection remains to be elucidated, as is also the case for gC−HS binding. However, the finding of a direct dependency on gC for HSV attachment to as well as infection of a CS-expressing cell line is intriguing in light of recent findings of gC requirement for replication in human skin implants in a mouse model (Moffat et al., 1998) and demonstration of CS expression in such tissues (Bode-Lesniewska et al., 1996; Sorrell et al., 1999; Zimmermann et al., 1994).

This work was supported by grants from the foundation ‘Glycoconjugates in Biological System’, the Swedish Medical Research Council (grant no. 11225), the Sahlgrens’ University Hospital Lakarutbildningsavtal and the Scandinavian Society for Antimicrobial Chemotherapy. We
thank Dorothy Spillmann for the gift of selectively desulfated heparin preparations and Maria Johansson for skilful technical assistance.

References


Trybala, E., Bergström, T., Svennerholm, B., Jeansson, S., Glorioso,}


Received 21 August 2001; Accepted 16 October 2001