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Structural and functional features of the polycationic peptide required for inhibition of herpes simplex virus invasion of cells

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Abstract

Glycoprotein C (gC) of herpes simplex virus type 1 (HSV-1) mediates initial virus contact with cells by binding to heparan sulfate (HS) chains. The synthetic peptide ^{137}GSRVQIRCRFRNSTR^{151} overlapping a major part of the HS-binding site of gC inhibited HSV-1 infection and, to some extent, HSV-2 infection of cells. Experiments on mutant, glycosaminoglycan-deficient cells as well as the binding assays involving peptide and purified cell surface components identified HS, and, to a lesser degree, chondroitin sulfate as sites of peptide activity. Anti-HSV-1 activity of the peptide was due to (i) partial inhibition of virus binding to cells and (ii) arresting the virions, which managed to attach to the cells in the presence of peptide, at a step of initial relatively weak binding. Analysis of the ionic-strength dependence of the peptide–HS and the virus–HS interactions revealed that the more efficient inhibition by the peptide of HSV-1 than HSV-2 infectivity was due to a relatively high affinity of HSV-2 for HS, a feature of importance in overcoming the peptide block. Mutational analysis of viral gC and peptide variants identified, apart from basic amino acids, two hydrophobic residues Ile^{142} and Phe^{146} as important in maintaining the specific affinity of peptide for HS and, hence, its anti-HSV activity. These results could contribute to the development of anti-HSV compounds that target initial events in the virus–cell interaction.

Keywords: HSV; Basic peptide; Heparan sulfate; Virus attachment; Glycoprotein C

1. Introduction

A number of pathogens (Rostand and Esko, 1997), including herpes simplex virus (HSV) (WuDunn and Spear, 1989), are known to adhere to the cell surface by interaction with heparan sulfate (HS) chains. The biological features of HS chains that make them well suited for this interaction include location at the cell periphery, abundant and ubiquitous expression, a strong negative charge load, and, most of all, the high degree of structural diversity of HS molecules expressed in various cells and tissues. Interaction of HSV type 1 (HSV-1) with cell surface HS chains is a complex process, and, apart from serving as receptor molecules for initial virus binding to cells (WuDunn and Spear, 1989), HS chains have, among other molecules, been reported to promote HSV-1 entry into the cell (Shukla et al., 1999). Of at least 11 kinds of envelope glycoproteins encoded by HSV-1, glycoprotein C (gC) was reported to be a major viral attachment protein (Herold et al., 1991), whereas gB was suggested to perform a corresponding function in mutant gC-negative HSV-1 (Herold et al., 1994). In a post-attachment step, interaction of gD with either tumor necrosis factor receptor-like protein (Montgomery et al., 1996), nectins (Geraghty et al., 1998) or HS chains that had been modified by 3-*O*-sulfation at an N-unsubstituted glucosamine residue (Liu et al., 1999; Shukla et al., 1999), was reported to trigger the virus entry into cells.

According to the most accepted opinion (Cardin and Weintraub, 1989), the HSV–HS interactions rely on multiple associations between the positively charged amino acid residues of the HS-binding sites of viral glycoproteins and the negatively charged sulfate/carboxylate groups of HS chain. Consequently, HSV infection of cells could be prevented at this stage by the two groups of oppositely charged compounds mimicking the viral and the HS components, respectively. The inhibition of HSV infection of cells by various HS-mimetics, including sulfated polysaccharides,

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polysulfonates, and other polyanions, is a well-studied phenomenon (for reviews, see Vaheri, 1964; De Clercq, 1993). In contrast, anti-HSV effects of basic proteins, peptides, and polycations, i.e. compounds mimicking the viral HS-binding components, have received limited attention (Hasegawa et al., 1994; Langeland et al., 1987, 1988). Although their effects on HSV infection are believed to be due to a competition with the viral attachment/entry proteins for binding to cell surface HS chains, it is intriguing that the attachment stage of viral infection is only moderately inhibited or in some cases unaffected or even enhanced by these compounds. Moreover, both types of HSV target HS chains as an initial receptor; however, HSV-1 infection of cells is usually more affected by polycations than HSV-2 infection (Langeland et al., 1988).

We have previously found that the polycationic peptide ^{137}GSRVQIRCRFRNSTR^{151} (Gly^{137}-Arg^{151}) overlapping a major part of the HS-binding site of HSV-1 gC (Trybala et al., 1994; Mardberg et al., 2001) inhibited virus infection of cells (Trybala et al., 1994). Here, we use variants of this peptide as well as HSV-1 mutants that carry corresponding alterations in gC to investigate the mode of peptide–cell and peptide–virus interaction, the aim being to identify features of this interaction that are essential for inhibition of viral infection of cells.

2. Experimental procedures

2.1. Cells and viruses

African green monkey kidney cells (GMK AH1; Gunalp, 1965) were propagated in Eagle's minimum essential medium supplemented with 2% newborn calf serum and 0.05% Primaton RT substance (Kraft Inc., Norwich, Conn., USA). Murine L, gro2C (Gruenheid et al., 1993), sog9 (Banfield et al., 1995), and sog9-EXT1 (McCormick et al., 1998) cells were kindly provided by Dr. Frank Tufaro, University of British Columbia (Vancouver). These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HSV-2 B4327UR strain (Jeansson and Molin, 1974), HSV-1 KOS 321 strain (Holland et al., 1983a), and its gC-negative derivative gC-101 (Holland et al., 1984), were used throughout the study.

2.2. Site-directed mutagenesis of HSV-1 gC

The assay was performed by using Promega's Altered Sites II In Vitro Mutagenesis System (Scandinavian Diagnostic Services, Falkenberg, Sweden), as previously described (Mardberg et al., 2001). Briefly, the pAltgC plasmid that contained an insert of 639 bp of HSV-1 gC DNA was used as a template for site-specific alterations. Mutagenesis primers (Scandinavian Gene Synthesis, Köping, Sweden) were designed to convert hydrophobic amino acids specified by codons 140, 142, and 146 to threonines. Template with confirmed alterations was subcloned into pGC, a plasmid that comprises the HSV-1 gC gene, and then cotransfected with gC-39 (a gC-null mutant) genomic DNA into vero cells. Mutant viruses with recombined gC protein were identified and purified to homogeneity by using an immunoreactive black plaque assay (Holland et al., 1983b).

2.3. Purification of virus particles and viral envelope glycoprotein gC

[*methyl*-^{3}H]Thymidine-labeled and unlabeled virions were purified from infectious culture media of GMK AH1 cells by centrifugation through a three-step discontinuous sucrose gradient (Karger and Mettenleiter, 1993; Trybala et al., 2000). Relative amounts of virions in preparations of purified virus were evaluated by comparing the densities of the major viral capsid protein (VP5), as described by WuDunn and Spear (1989). The viral gC was purified by immunoaffinity chromatography, as described previously (Trybala et al., 2000).

2.4. Peptide synthesis

Based on the sequence data for gC of HSV-1 (Frink et al., 1983), the Gly^{137}-Arg^{151} peptide and its modifications were synthesized by using the Fmoc synthesis protocol and analyzed by reverse-phase HPLC and/or ion spray mass spectrometry (Chiron Mimotopes, Victoria, Australia). Peptides contained a free amino group and amide group at the N- and C-terminus, respectively. Peptides were dissolved in MilliQ water and the stocks (0.5–2.5 mM) were stored at −20 °C. The cytotoxicity of peptides was measured by the intake of neutral red dye following incubation of the cells with a peptide for 24 h at 37 °C.

2.5. Isolation of glycosaminoglycan chains

Nearly confluent monolayers of GMK AH1 cells were washed with low-sulfate Eagle's medium (10% original $MgSO_4$ concentration) and then incubated for 48 h with the same medium supplemented with $Na_2{}^{35}SO_4$ at 50 μCi/ml (NEN Life Science Products; specific activity 1325 Ci/mmol), 10% fetal calf serum, and antibiotics. Cell-associated HS chains were purified by the method of Lyon et al. (1994). Chondroitin sulfate (ChS) chains were prepared in a similar manner except that samples of [^{35}S]-labeled material were treated with heparinase I (heparin lyase I, EC 4.2.2.7, Sigma) to degrade HS chains. The specificity of the glycosaminoglycan preparations was tested, as described by Mardberg et al. (2002).

2.6. Binding of glycosaminoglycans to peptides, gC molecules, and HSV-1 virions

Equal quantities of peptides, purified gC species (adjustment according to protein content), or purified mutant

virions (comparison based on the VP5) were diluted in 0.2 ml of PBS supplemented with 0.05% BSA and then mixed with approximately 5000 c.p.m. of GMK AH1 [^{35}S]-labeled HS or ChS. Following incubation of the mixtures for 2 h at room temperature, the amounts of bound glycosaminoglycan were determined by the nitrocellulose membrane filtration method (Maccarana and Lindahl, 1993). Some experiments were carried out in the presence of higher concentrations of NaCl than those found in PBS.

2.7. Binding of peptide to HSV virions

The Gly^{137}-Arg^{151} peptide, biotinylated at the N-terminal ending, was incubated with streptavidin-coated agarose beads (Pierce, Rockford, IL, USA) for 2 h at room temperature in PBS supplemented with 0.05% BSA (PBS–BSA). Subsequently, 0.4 ml vol. of peptide- or sham-coated beads were transferred to the columns and [^{3}H]thymidine-labeled HSV particles in PBS–BSA were circulated through the columns. The beads were washed four times with 1 ml of PBS–BSA and then eluted with 1 ml of 5% SDS in PBS–BSA.

2.8. HSV attachment–inhibition assay

GMK AH1 cells in 24-well plates were washed with PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-A), and then pretreated with PBS-A-containing 1% bovine serum albumin for 1 h at room temperature. The cells were washed once with PBS-A and 225 μl of the same buffer supplemented with various concentrations of peptide were added. Following incubation for 15 min at 4 °C, 5 μl of radiolabeled virus was added, and virus adsorption to cells was allowed under moderate agitation for 2 h at 4 °C. Subsequently, the cells were washed three times with PBS-A, lysed with 0.2 ml of PBS-containing 5% SDS, and finally transferred to scintillation vials for quantification of radioactivity. Elution of preadsorbed HSV-2 with hypertonic NaCl was carried out in a similar manner, except that following 45-min period of virus attachment to cells in the presence of peptide (50 μM), the cells were triple washed with 0.2 ml of PBS for 3 min under moderate agitation, and then stepwise eluted in an identical manner with hypertonic NaCl solutions. The latter were made by adjusting the NaCl content in PBS with a 2 M solution of NaCl in phosphate buffer.

2.9. Viral plaque assays

Effect of synthetic peptides on HSV infectivity was tested as follows. Confluent monolayers of 3-day-old GMK AH1 cells in 6-well plates were washed twice with 2 ml of Eagle's medium, and 1 ml portions of the same medium containing indicated concentrations of peptide were added. Following incubation for 15 min at 37 °C, approximately 200 plaque forming units (p.f.u.) of the virus were added and left for adsorption for a further 1 h at 37 °C. The cells were then washed twice with 2 ml of Eagle's medium and overlaid with 3 ml of 1% methylcellulose solution in the same medium. After incubation for 3 days at 37 °C, the cells were stained with 1% solution of crystal violet to visualize the viral plaques.

To study the ionic-strength dependence of the viral interaction with cells, confluent, dense, monolayers of 3-day-old GMK AH1 cells in 6-well plates were precooled for 30 min at room temperature and for 1 h at 4 °C. The cells were washed twice with 1 ml of cold Hanks medium modified to contain the indicated concentrations of NaCl, and approximately 200 p.f.u. of the virus diluted in the medium of the same ionic strength were added and left for adsorption for 20 min at 4 °C. The cells were then washed with 1 ml of the same medium as used for the virus adsorption and two extra times with 2 ml of normal Hanks medium. Subsequently, the cells were overlaid with 1% methylcellulose solution and incubated for 3 days at 37 °C for viral plaques to develop. A solution of 2 M NaCl in Hanks medium was added to normal Hanks medium to achieve the required NaCl concentrations.

2.10. NMR spectroscopy

NMR sample contained the Gly^{137}-Arg^{151} peptide (at 1.8 mM) in 90% H_2O and 10% $^{2}H_2O$. NMR measurements were carried out either at 25 or 10 °C on a Varian UNITY plus-500 and Varian XL-300 spectrometers. Two-dimensional NMR data were processed using VNMR software. The assignments of proton signals were accomplished using TOCSY and ROESY data obtained at 10 °C. The mixing time of ROESY was 0.1 s. For analysis of peptide conformation in the presence of heparin, the Gly^{137}-Arg^{151} peptide at 1.2, 1, and 0.5 mM was mixed with either 22- to 24-mer heparin oligosaccharide (at 1 mM), 24- to 26-mer heparin oligosaccharide (0.04 mM) supplemented with 0.25 or 4 M NaCl in 10 mM acetate buffer, or 10-mer heparin fragment in 0.5 M NaCl.

3. Results

3.1. Differential effects of the Gly^{137}-Arg^{151} peptide on HSV-1 and HSV-2 infection of cells

The effect of the Gly^{137}-Arg^{151} peptide on HSV-1 and HSV-2 infection of GMK AH1 cells is shown in Fig. 1A. The concentration of peptide that reduced by 50% (IC_{50}) the number of HSV-1 plaques was 19 μM (Fig. 1A). Under the same experimental conditions, the peptide at the two highest concentrations tested reduced HSV-2 infectivity to a lesser extent than that of HSV-1. The concentration of peptide that was toxic for GMK AH1 cells (CC_{50}) was 215 μM (neutral red intake method; data not shown), thus 11 times exceeding its IC_{50} value. The scrambled (control) variant of the Gly^{137}-Arg^{151} peptide did not inhibit HSV-1 infectivity even when tested at 125 μM (Fig. 1A, dashed line). The

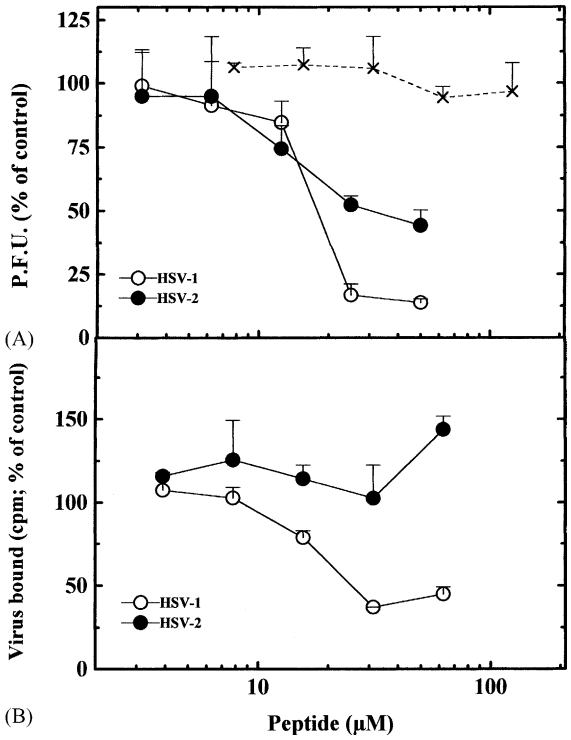

Fig. 1. Effect of the Gly^{137}-Arg^{151} peptide on infectivity (A) and binding to cells (B) of HSV-1 and HSV-2. GMK AH1 cells were incubated with the peptide for 15 min before addition of the virus. The dashed line in panel A represents the effect of control, scrambled variant of the Gly^{137}-Arg^{151} peptide on HSV-1 KOS 321 infectivity. Values shown are averages of two (panel B) or four (panel A) individual determinations from two separate experiments.

Gly^{137}-Arg^{151} also inhibited infectivity of gC-negative variants of HSV-1 and HSV-2 with the IC_{50} values of 14 and 10 μM, respectively (data not shown). To determine whether the inhibitory activity of the Gly^{137}-Arg^{151} peptide was due to interference with the virus binding to cells, the effect of Gly^{137}-Arg^{151} on attachment to cells of purified radiolabeled virus was tested. The peptide partially inhibited the binding of HSV-1 but not HSV-2 (Fig. 1B).

3.2. Site(s) of peptide activity

Having determined that the Gly^{137}-Arg^{151} interfered with HSV-1 binding (Fig. 1B) and knowing that cell surface HS chains or in their absence ChS chains provide receptor sites for binding of HSV, we investigated whether these molecules were important for peptide activity. To this end, the effect of Gly^{137}-Arg^{151} on HSV-1 infectivity in mouse L cell mutants that differ in the expression of glycosaminoglycans was tested. Parental L cells express HS and ChS chains, sog9 cells are both HS- and ChS-deficient, gro2C cells produce ChS but not HS chains, whereas sog9-EXT1 cells express HS only. The Gly^{137}-Arg^{151} peptide reduced HSV-1 infection in sog9-EXT-1, L and, to some extent in gro2C cells (Fig. 2A). In contrast, HSV-1 infection of HS-

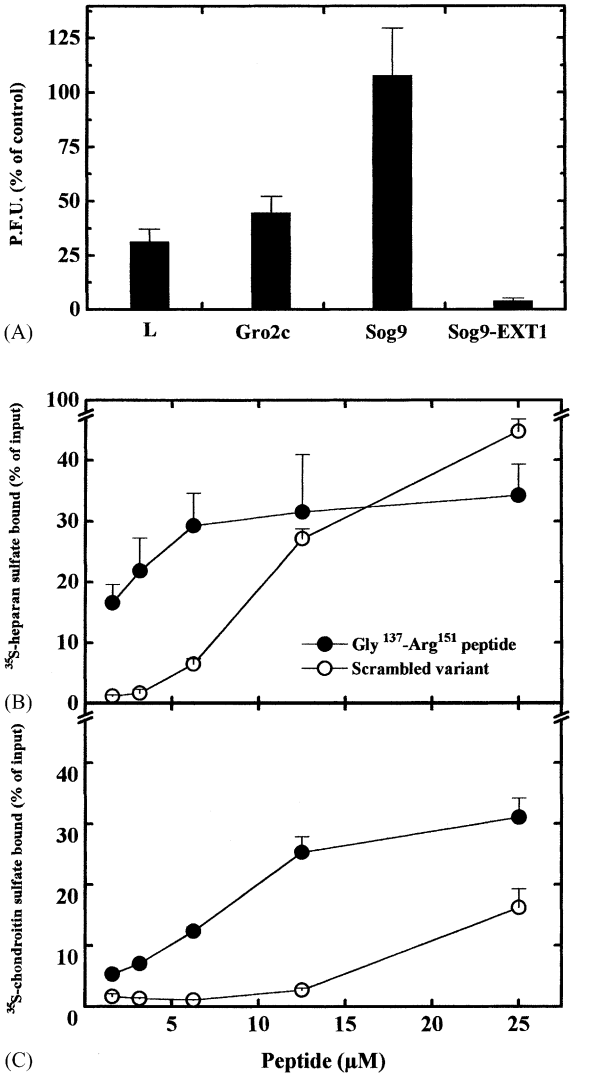

Fig. 2. Site of peptide activity. (A) Effect of the Gly^{137}-Arg^{151} peptide on HSV-1 infectivity in glycosaminoglycan-deficient mutants of mouse L cells. Parental (L), HS-deficient (Gro2c), HS- and ChS-deficient (Sog9) cells, as well as sog9 cells with restored HS expression (Sog9-EXT1) were incubated with the peptide (at 62.5 μM) for 15 min before addition of the virus. Two separate experiments were carried out in duplicate with each cell line. (B) and (C) HS- and ChS-binding capabilities of the Gly^{137}-Arg^{151} peptide and its scrambled analogue. GMK AH1 cell-specific, [^{35}S]-labeled HS (B) or ChS (C) were incubated for 2 h at room temperature with peptides. Bound HS or ChS chains were trapped on nitrocellulose filters. Values shown are averages of four individual determinations from two separate experiments.

and ChS-deficient sog9 cells, was not affected by peptide suggesting that the anti-HSV-1 activity of the Gly^{137}-Arg^{151} could be related to interference with interaction between HSV and HS/ChS. To investigate whether the Gly^{137}-Arg^{151} can indeed interact with HS and ChS chains, a direct binding assay was performed (Fig. 2B). The Gly^{137}-Arg^{151} bound purified HS chains. The scrambled variant of the Gly^{137}-Arg^{151} peptide was also able to interact with HS; however, in contrast to the Gly^{137}-Arg^{151} it bound negligible amounts of HS at relatively low peptide-to-HS ratios.

Table 1
Binding of HSV-1 and HSV-2 particles to the Gly^{137}-Arg^{151} peptide-coated beads[a]

Fraction of [^{3}H] label	HSV-1		HSV-2	
	Peptide	Control	Peptide	Control
Unadsorbed to beads	57.9[b]	77.0	8.1	36.4
Removed by washing 1	15.5	20.7	8.9	44.9
Removed by washing 2	0.8	0.4	0.9	0.6
Removed by washing 3	0.5	0.1	0.7	0.2
Removed by washing 4	0.5	0.1	0.6	0.1
Eluted from beads	19.8	1.4	76.3	17.3
Remained on beads	5.0	0.3	4.5	0.5

[a] Following incubation of the peptide-coated or control beads with [^{3}H]thymidine-labeled HSV-1 or HSV-2, the columns were washed with PBS and then eluted with 5% SDS in PBS.
[b] Mean of duplicate counting expressed as % of total c.p.m. recovered.

At high concentrations of the scrambled peptide, its binding capabilities exceeded those of parental Gly^{137}-Arg^{151} peptide. Both peptides exhibited less efficient interaction with ChS (Fig. 2C) than HS (Fig. 2B) chains.

Results presented in Fig. 1 showed that when the virus was added to cells in the presence of high concentrations of the peptide there was more efficient inhibition of HSV-1 and HSV-2 infectivity (Fig. 1A) than of binding of these viruses to cells (Fig. 1B). However, when an excess of the peptide (added at 50 μM) was removed from cells prior to the virus addition, there were 81.2 and 36.0% reductions in HSV-1 and HSV-2 binding to cells (data not shown), i.e. a more efficient inhibition than those shown in Fig. 1B. This suggests that an excess of the peptide might stimulate the virus binding to cells, and that this stimulation was greater for HSV-2 than HSV-1 virions. We then investigated whether the Gly^{137}-Arg^{151} could interact with the virus particles. The biotinylated Gly^{137}-Arg^{151} was preadsorbed on the streptavidin–agarose beads and then the [^{3}H]thymidine-labeled HSV-2 was run through the column (Table 1). Although the control beads (without peptide) demonstrated some unspecific adsorption, particularly of HSV-2 virions, peptide-coated beads bound considerably more [^{3}H]-labeled HSV-2 than HSV-1 particles (Table 1; compare the eluted fractions). This finding suggests that, at relatively high concentrations, the Gly^{137}-Arg^{151} might enhance unspecific binding, particularly of HSV-2, to cells (Fig. 1B).

3.3. Ionic-strength dependence of the peptide–HS interaction as criterion for anti-HSV activity

The results presented in Fig. 2B indicated that both the Gly^{137}-Arg^{151} and its scrambled variant were capable of binding HS; however, only the Gly^{137}-Arg^{151} peptide inhibited HSV-1 infection of cells (Fig. 1A). This observation encouraged us to investigate whether some parameters of peptide–HS interaction could be essential for the Gly^{137}-Arg^{151} ability to block HSV infection. To this end,

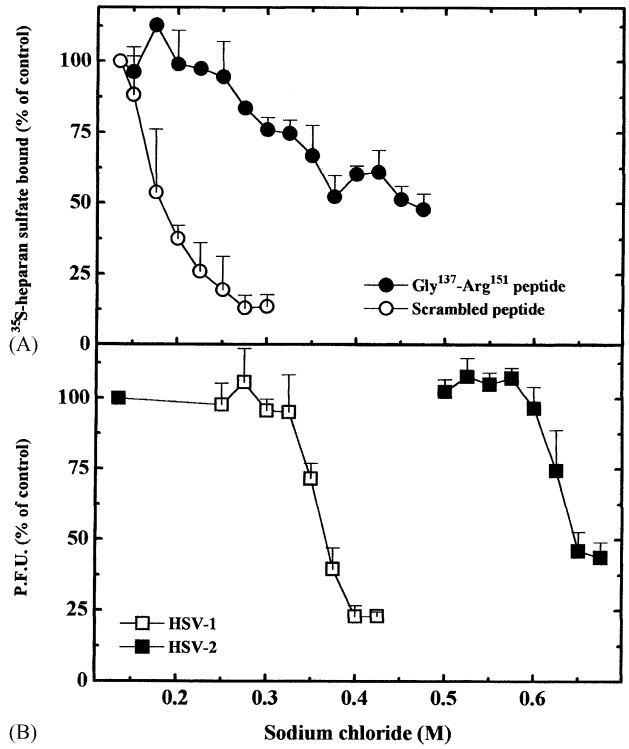

Fig. 3. (A) The ionic-strength dependence of interaction between the Gly^{137}-Arg^{151} peptide and HS chains. GMK AH1 cell-specific, [^{35}S]-labeled HS was incubated for 2 h at room temperature with peptides in the presence of indicated concentrations of sodium chloride. Bound HS chains were trapped on nitrocellulose filters. The amount of HS bound at isotonic NaCl concentration (0.137 M NaCl) was accepted as control. Values shown are averages of two individual determinations from two separate experiments. (B) The ionic-strength dependence of interaction between HSV-1/HSV-2 and GMK AH1 cells. Experiments were carried out on confluent, dense, monolayers of cells at 4 °C (cold room). Viruses were incubated with the cells for 20 min at 4 °C in the presence of specific concentrations of sodium chloride in the medium. The cells were then washed and incubated at 37 °C for viral plaques to develop. Values shown are averages of four individual determinations from two separate experiments.

we tested the sensitivity of the peptide–HS interaction to the ionic strength, which is a widely used criterion for indirect evaluation of the binding affinities of different proteins for heparin/HS. The interaction of scrambled peptide with HS chains was very sensitive to increased ionic strength of the medium with 50% reduction of binding at 0.18 M NaCl (Fig. 3A). In contrast, the Gly^{137}-Arg^{151} activity was more resistant to increased ionic strength, and substantial amounts of HS were bound even at 0.475 M NaCl. Thus, the results obtained with scrambled peptide suggested that an ability to interact with HS was not sufficient to inhibit HSV-1 infection, and that a certain affinity of the binding might be required for anti-HSV activity to occur.

To relate peptide data with those of virus infectivity, but most of all to search for an explanation of why the Gly^{137}-Arg^{151} peptide more efficiently inhibited HSV-1 than HSV-2 infection of cells (see Fig. 1A), the effect of

increased ionic strength on attachment of these viruses to GMK AH1 cells was assayed. It should be noted that this experiment required exposure of cells to hypertonic concentrations of NaCl that might be toxic for cells. Our data indicate that the highest concentration of NaCl employed in this experiment, i.e. 0.675 M was virtually non-toxic for GMK AH1, HEp-2, HeLa, and epithelial buccal cells but toxic for mouse L and gro2C cells when present on confluent monolayers of these cells for 40 min at 4 °C (neutral red intake method). The results (Fig. 3B) showed that HSV-2 bound with higher affinity than HSV-1 to cell surface HS/ChS as approximately 0.65 and 0.35 M NaCl were required for 50% reduction in the number of HSV-2 and HSV-1 plaques, respectively. These results suggest that the inability of the scrambled peptide to inhibit HSV-1 infectivity (Fig. 1A) could be related to its lower affinity than HSV-1 virions (Fig. 3A and B) for cell surface HS. Likewise, the affinity of the Gly^{137}-Arg^{151} for HS chains (Fig. 3A) was on the average higher than HSV-1 but lower than HSV-2 (Fig. 3B), and the peptide inhibited HSV-1 infectivity but had only moderate effect on HSV-2 infectivity (Fig. 1A).

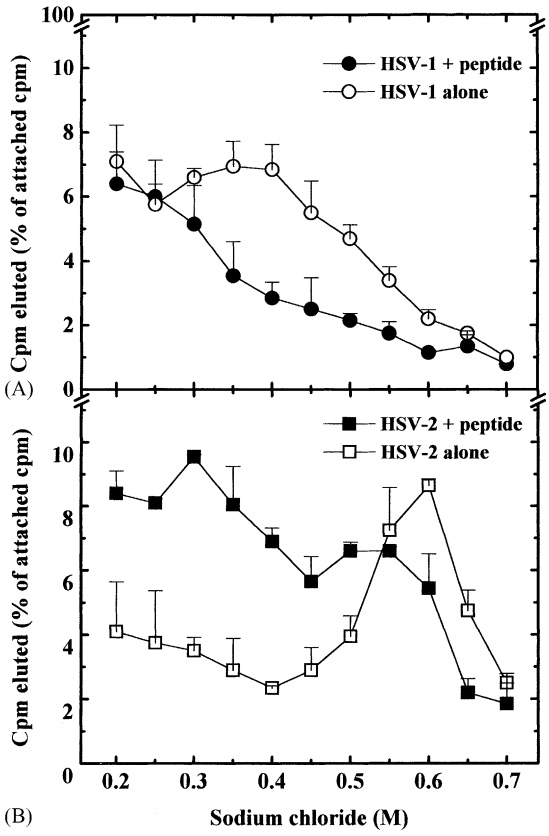

Fig. 4. Modulation of HSV binding to cells by the Gly^{137}-Arg^{151} peptide. Purified, [^{3}H]thymidine-labeled HSV-1 (A) or HSV-2 (B) particles, preadsorbed to GMK AH1 cells in the presence of Gly^{137}-Arg^{151} peptide (50 μM), were stepwise eluted with increasing concentrations of NaCl. Values shown are averages of two replicates from two separate experiments.

To further investigate the mechanism of anti-HSV activity of the peptide, HSV-1 and HSV-2 virions were adsorbed to cells in the presence of peptide and then stepwise eluted with increasing concentrations of NaCl (Fig. 4). The Gly^{137}-Arg^{151} decreased the affinity of the salt-sensitive HSV-1 binding to cells as most of the virus particles that managed to bind to cells in the presence of Gly^{137}-Arg^{151} eluted at lower NaCl concentrations than virions bound in the absence of peptide (Fig. 4A). A lesser total amount of virus eluted in the presence of peptide than in its absence could be attributed to the fact that the presented data concerned the virus that resisted the peptide-mediated attachment blockage, in which the relative contribution (%) of the salt-resistant fraction was proportionally enlarged. HSV-2 virions, adsorbed to cells in the absence of peptide, eluted over a wide range of NaCl concentrations with a peak at 0.6 M NaCl (Fig. 4B). In contrast, a proportion of HSV-2 adsorbed in the presence of peptide, eluted at low NaCl concentration, indicative of relatively "loose", low-affinity binding of the virus to cells. Note, however, that a fraction of labeled HSV-2 particles demonstrated in the presence of peptide a relatively tight binding, as judged by their elution at 0.45–0.6 M NaCl (Fig. 4B). These results suggest that, in addition to the effects shown previously (Fig. 1), the peptide can arrest HSV particles at the early, "loose" phase of the attachment step.

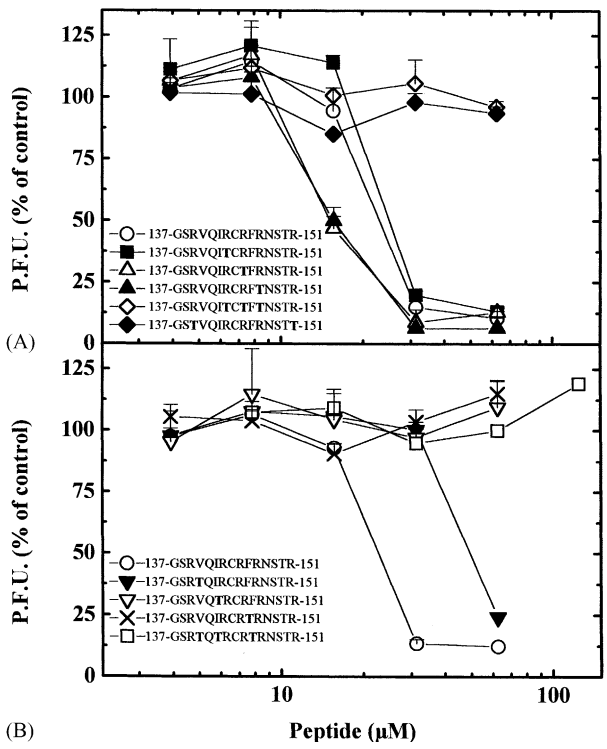

Fig. 5. Importance of charged (A) and hydrophobic (B) residues of the Gly^{137}-Arg^{151} peptide for its ability to inhibit HSV-1 KOS 321 infection of cells. For an explanation, see Fig. 1A.

3.4. Amino acid residues important for anti-HSV activity of peptide

To identify structural features of the peptide that are important for its anti-HSV-1 activity, several Gly^{137}-Arg^{151} variants carrying single or multiple substitution(s) of threonine for basic amino acid residues, i.e. either Arg^{139}, Arg^{143}, Arg^{145}, Arg^{147}, or Arg^{151}, were tested. Substitution of threonine for either Arg^{143}, Arg^{145}, or Arg^{147} had little or no effect on the biological activity of the peptide (Fig. 5A), however, simultaneous replacement of these three residues with threonines abrogated peptide activity. The same appeared to be true when the two flanking arginines Arg^{139} and Arg^{151} were simultaneously changed to threonines. In sharp contrast to the minor effects exerted by individual replacement of positively charged residues, substitution of threonine for hydrophobic amino acids, i.e. either Ile^{142} or Phe^{146}, significantly affected the ability of the peptide to inhibit virus infection of GMK AH1 cells (Fig. 5B). The "hydrophilic" analogue of the Gly^{137}-Arg^{151} sequence, i.e. the peptide carrying simultaneous substitutions of threonine for Val^{140}, Ile^{142}, and Phe^{146} was inactive even at a peptide concentration of 125 μM.

In addition, we sought to examine whether these specific hydrophobic residues were relevant for the ability of HSV-1 to bind HS. To this end, mutants in gC of HSV-1 that carried a single substitution of threonine for either Val^{140}, Ile^{142}, or Phe^{146} were prepared. Both mutated virions and gCs purified from respective virus mutants were tested for their ability to bind HS chains (Fig. 6). Mutant viruses altered at either Ile^{142} or Phe^{146} bound significantly less HS than parental KOS 321 strain (Fig. 6A). The same appeared true when gC isolated from Ile^{142}Thr or Phe^{146}Thr mutant was assayed (Fig. 6B). These results confirmed that certain hydrophobic amino acids that were critical for antiviral activity of the Gly^{137}-Arg^{151} were also important for efficient interaction of HSV-1 virions and viral gC with HS.

To determine how hydrophobic residues may contribute to the efficient binding to HS and thereby to the expression of biological activities of the Gly^{137}-Arg^{151} peptide, we first investigated the structure forming functions of these residues. Analysis of the Gly^{137}-Arg^{151} structure by NMR technique

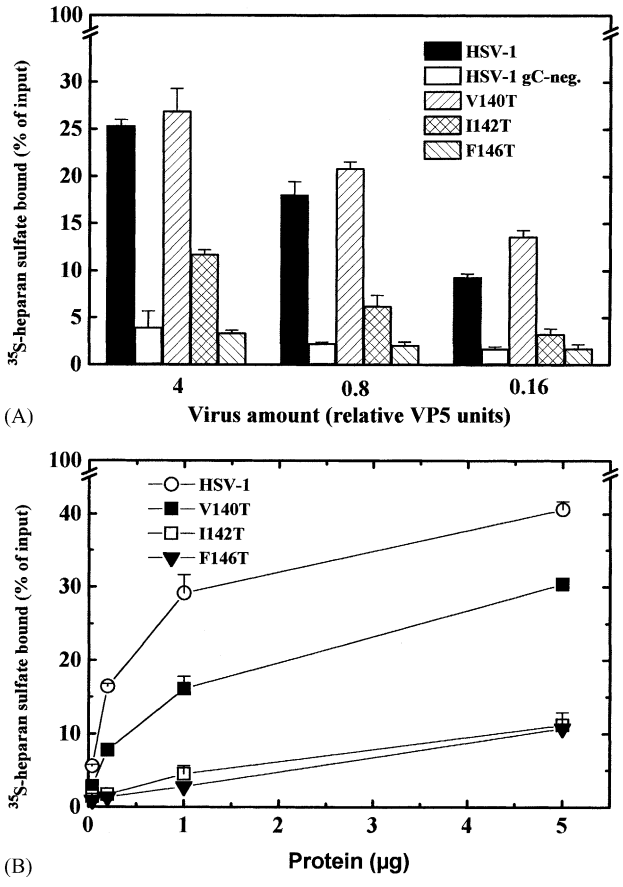

Fig. 6. HS-binding capabilities of HSV-1 KOS 321 mutant viruses and isolated gC proteins altered at specific hydrophobic residues. GMK AH1 cell-specific, [^{35}S]-labeled HS was incubated for 2 h at room temperature with the purified viral mutants, including gC-negative variant (A) or isolated gC proteins (B). Bound HS chains were trapped on nitrocellulose filters. Values shown are averages of four individual determinations from two separate experiments.

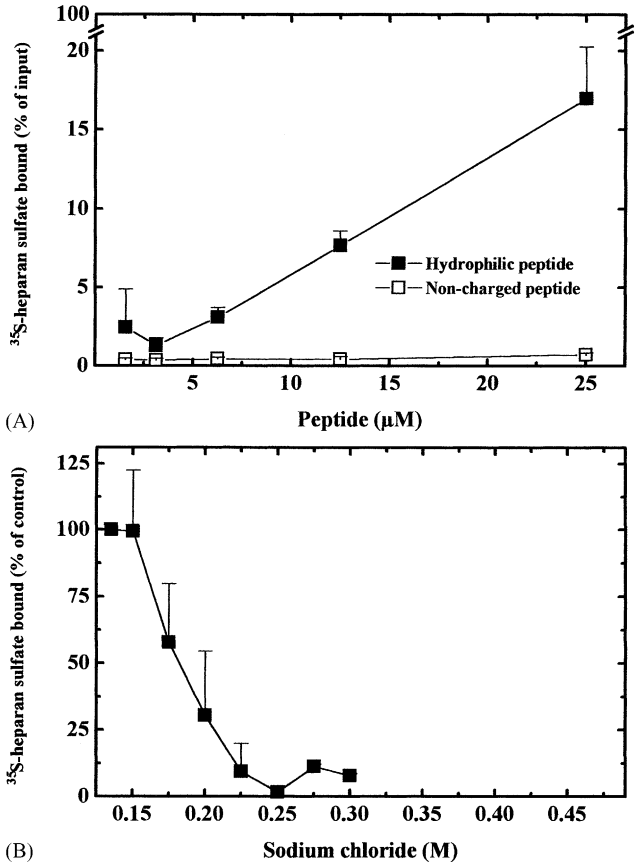

Fig. 7. HS-binding capabilities of the hydrophilic and non-charged analogues of Gly^{137}-Arg^{151} peptide (A) and ionic-strength dependence of the hydrophilic peptide–HS interaction (B). For explanations, see Figs. 2B and 3A. Values shown are averages of four (panel A) or two (panel B) individual determinations from two separate experiments.

revealed that it did not adopt any specific, structured form in aqueous solution, although the peptide might be extended (data not shown). Analysis of peptide conformation in the presence of heparin (or its fragments) was hampered by a rapid formation of aggregates.

To further investigate how hydrophobic residues would affect interaction between the Gly^{137}-Arg^{151} peptide and glycosaminoglycans, the "hydrophilic" variant of the Gly^{137}-Arg^{151} sequence (for an explanation, see above) and the "non-charged" Gly^{137}-Arg^{151}, i.e. the peptide carrying simultaneous substitutions of glycine for all arginine residues, were tested for ability to bind HS. The "hydrophilic" but not "non-charged" peptide was capable of binding HS (Fig. 7A), although its HS-binding capability was less pronounced than that of parental peptide (compare data in Figs. 2B and 7A). The binding of HS by "hydrophilic" peptide began to decrease already at 0.175 M NaCl (Fig. 7B). These results demonstrated that in contrast to basic residues, substitution for hydrophobic amino acids decreased but did not abrogate the ability of peptide to bind HS, and that this binding was relatively weak as judged from the ionic-strength dependence experiment.

4. Discussion

We attempted to define the molecular basis behind the inhibition of HSV infection of cells by the Gly^{137}-Arg^{151}, a peptide that overlaps a major part of the HS-binding site of HSV-1 gC (Trybala et al., 1994; Mardberg et al., 2001). Experiments on mutant glycosaminoglycan-deficient cells and the direct binding assay involving peptide and isolated glycosaminoglycan chains identified cell surface HS as the major site of antiviral activity of the Gly^{137}-Arg^{151}. In addition, these experiments revealed that the cell surface ChS chains could serve as a substitute, low-affinity ligand for the peptide.

The Gly^{137}-Arg^{151} inhibited infection of cells by HSV-1, the gC-negative mutants of HSV-1 and HSV-2, and, to lesser degree, infectivity of wild-type HSV-2. In this respect, the activity of peptide resembled effects of polycationic substances polylysine and neomycin that also inhibited infectivity of HSV-1, however, in some cell lines, such as BHK cells, HSV-2 was unaffected (Campadelli-Fiume et al., 1990; Langeland et al., 1987, 1988, 1990; Oyan et al., 1993). This relative resistance of HSV-2 to polycationic compounds was found to be mediated by the gC component of the virus. We have recently observed that the binding of HSV-2 to cells was more resistant to increased ionic strength than that of HSV-1, and that this phenomenon was gC dependent (Trybala et al., 2000). Here, we identified the ionic-strength dependence of peptide–HS and the virus–HS interaction as an important criterion for predicting antiviral activity of the peptide and explaining its differential effects on HSV-1 and HSV-2 infections. According to the most accepted opinion, different HS-binding proteins, including the virus attachment components, bind to different but specific combinations of sulfate groups or other determinants of HS. However, all these motifs are likely to occur within the overlapping stretches of HS chain (the so-called sulfated domains) and, therefore, can be affected by different polycations. In such scenario, in addition to the specificity of interaction between polycationic inhibitor and HS, its affinity for HS seems to be an important antiviral determinant. Different polycations, including peptides and polycationic HS-binding sites, of the viral proteins demonstrate distinct affinity (ionic-strength dependence) of their binding to HS chains; and according to the macromolecule–polyelectrolyte interaction theory (Manning, 1978; Record et al., 1978), ligands that are relatively resistant to increased NaCl content (the high-affinity ligands) can displace the molecules that are more sensitive to NaCl in their binding to HS chains. This competition is also dependent upon the concentration of the compound used. Analysis of ionic-strength dependence of binding of whole HSV virions to cells revealed that the NaCl concentrations that reduced binding by 50% were 0.35 M for HSV-1 and 0.65 M for HSV-2. Thus, to inhibit HSV-1, a compound that is able to interact with HS in the presence of >0.35 M NaCl would be required. Note that the Gly^{137}-Arg^{151} peptide (with a 50% maximum binding to HS at ~0.45 M NaCl), but not its scrambled version (50% maximum binding to HS at ~0.18 M NaCl), fulfilled this criterion. Moreover, a compound that is able to interact with HS at >0.65 M NaCl would be needed to inhibit HSV-2 infection. Nonetheless, upon defining the HS-binding site of HSV-2 gC, it would be of interest to challenge this interpretation with peptides specific for the HS-binding sequences of this virus.

Our results demonstrated that the partial inhibition of the virus binding and/or arresting the viral binding at its early, "loose" phase were principally responsible for anti-HSV-1 activity of the peptide. In contrast, due to the high affinity of HSV-2 for cell surface HS, attachment of this virus to cells was not inhibited by Gly^{137}-Arg^{151}. In the presence of the peptide, HSV-2 virions bound either "loosely" to cells or completely overcame the peptide block. In addition, we have observed that the Gly^{137}-Arg^{151} peptide was able to interact with HSV-2 particles and, to lesser extent, HSV-1 particles, thus raising a possibility that the peptide might cross-link the virus particles and the cell surfaces. At present, it is unclear whether the "loose" virus binding to cells observed in the presence of peptide could be due to peptide-mediated cross-linking of virions to cells. It was reported that the polycationic compounds polylysine and/or neomycin enhanced infectivity of gC-negative mutants of pseudorabies virus (Zsak et al., 1990), bovine herpes virus 1 (Liang et al., 1991), and HSV-2 (Campadelli-Fiume et al., 1990). Interaction of polycations with both the viral particle and cell surface might well explain these effects.

Studies on the rational de novo design or optimization of the existing heparin/HS-binding peptides (e.g. Fromm et al., 1995, 1997; Jayaraman et al., 2000; Verrecchio

et al., 2000) demonstrated that specific spacing of positively charged amino acids and the size of the peptide were important for binding to heparin/HS. In addition to these requirements, our data showed that hydrophobic/aromatic amino acids, at specific locations relative to basic amino acids, were critical for anti-HSV-1 activity. This finding did not appear to be only a "peptide-related" phenomenon, as identical point-mutations introduced into HSV-1 gC confirmed that specific hydrophobic residues, especially Ile^{142} and Phe^{146}, were important for efficient binding of whole virions or isolated viral gC to HS. Unlike the basic amino acids, hydrophobic/aromatic residues were not essential for the initial contact with HS chains, since the "hydrophilic" peptide retained some HS-binding capability. Instead, these residues were critical in maintaining a specific affinity of the peptide for HS and hence, its anti-HSV activity.

Certain naturally occurring antimicrobial proteins are of polycationic nature (e.g. lactoferrin), and our work on anti-HSV activity of the Gly^{137}-Arg^{151} peptide may contribute to the development of drugs mimicking this class of antimicrobial compounds. In particular, identification of the glycosaminoglycan-binding affinity of a polycation as an antiviral criterion together with the possibility of modulation of this feature by a hydrophobic/aromatic component may help in the rational design of candidate compounds. An association of the binding affinity criterion with anti-HSV effects has also been suggested for certain polyanionic compounds. The poor protection of mice against HSV-2 infection by dextran sulfate has been attributed to a weak and reversible interaction of this polyanion with the virus particle (Neyts and De Clercq, 1995). In contrast, lambda carrageenans bind irreversibly to HSV virions and were found to confer complete protection of mice against HSV-2 infection (Zacharopoulos and Phillips, 1997).

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References

Banfield, B.W., Leduc, Y., Esford, L., Schubert, K., Tufaro, F., 1995. Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. J. Virol. 69, 3290–3298.

Campadelli-Fiume, G., Stirpe, D., Boscaro, A., Avitabile, E., Foa-Tomasi, L., Barker, D., Roizman, B., 1990. Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. Virology 178, 213–222.

Cardin, A.D., Weintraub, H.J., 1989. Molecular modeling of protein–glycosaminoglycan interactions. Arteriosclerosis 9, 21–32.

De Clercq, E., 1993. Antiviral agents: characteristic activity spectrum depending on the molecular target with which they interact. Adv. Virus Res. 42, 1–55.

Frink, R.J., Eisenberg, R.J., Cohen, G.H., Wagner, E.K., 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. J. Virol. 45, 634–647.

Fromm, J.R., Hileman, R.E., Caldwell, E.E., Weiler, J.M., Linhardt, R.J., 1995. Differences in the interaction of heparin with arginine and lysine and the importance of these basic amino acids in the binding of heparin to acidic fibroblast growth factor. Arch. Biochem. Biophys. 323, 279–287.

Fromm, J.R., Hileman, R.E., Caldwell, E.E., Weiler, J.M., Linhardt, R.J., 1997. Pattern and spacing of basic amino acids in heparin binding sites. Arch. Biochem. Biophys. 343, 92–100.

Geraghty, R.J., Krummenacher, C., Cohen, G.H., Eisenberg, R.J., Spear, P.G., 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science 280, 1618–1620.

Gruenheid, S., Gatzke, L., Meadows, H., Tufaro, F., 1993. Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans. J. Virol. 67, 93–100.

Gunalp, A., 1965. Growth and cytopathic effect of rubella virus in a line of Green Monkey kidney cells. Proc. Soc. Exp. Biol. Med. 118, 185–190.

Hasegawa, K., Motsuchi, W., Tanaka, S., Dosako, S., 1994. Inhibition with lactoferrin of in vitro infection with human herpes virus. Jpn. J. Med. Sci. Biol. 47, 73–85.

Herold, B.C., WuDunn, D., Soltys, N., Spear, P.G., 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. 65, 1090–1098.

Herold, B.C., WuDunn, D., Visalli, R.J., Susmarski, N., Brandt, C., Spear, P.G., 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulfate and glycoprotein B. J. Gen. Virol. 75, 1211–1222.

Holland, T.C., Marlin, S.D., Levine, M., Glorioso, J., 1983a. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. J. Virol. 45, 672–682.

Holland, T.C., Sandri-Goldin, R.M., Holland, L.E., Marlin, S.D., Levine, M., Glorioso, J., 1983b. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J. Virol. 46, 649–652.

Holland, T.C., Homma, F.L., Marlin, S.D., Levine, M., Glorioso, J., 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. 52, 566–574.

Jayaraman, G., Wu, C.W., Liu, Y.J., Chien, K.Y., Fang, J.C., Lyu, P.C., 2000. Binding of a de novo designed peptide to specific glycosaminoglycans. FEBS Lett. 482, 154–158.

Jeansson, S., Molin, L., 1974. On the occurrence of genital herpes simplex virus infection. Clinical and virological findings and relation to gonorrhoea. Acta Derm. Venereol. 54, 479–485.

Karger, A., Mettenleiter, T.C., 1993. Glycoproteins gIII and gp50 play dominant roles in the biphasic attachment of pseudorabies virus. Virology 194, 654–664.

Langeland, N., Moore, L.J., Lillehaug, J.R., Haarr, L., 1987. Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. J. Virol. 61, 3388–3393.

Langeland, N., Moore, L.J., Holmsen, H., Haarr, L., 1988. Interaction of polylysine with the cellular receptor for herpes simplex virus type 1. J. Gen. Virol. 69, 1137–1145.

Langeland, N., Oyan, A.M., Marsden, H.S., Cross, A., Glorioso, J.C., Moore, I.J., Haarr, L., 1990. Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. J. Virol. 64, 1271–1277.

Liang, X.P., Babiuk, L.A., van Drunen Littel-van den Hurk, S., Fitzpatrick, D.R., Zamb, T.J., 1991. Bovine herpesvirus 1 attachment to permissive

cells is mediated by its major glycoproteins gI, gIII and gIV. J. Virol. 65, 1124–1132.

Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., Rosenberg, R.D., 1999. Heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-3A sulfates N-unsubstituted glucosamine residues. J. Biol. Chem. 274, 38155–38162.

Lyon, M., Deakin, J.A., Mizuno, K., Nakamura, T., Gallagher, J.T., 1994. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J. Biol. Chem. 269, 11216–11223.

Maccarana, M., Lindahl, U., 1993. Mode of interaction between platelet factor 4 and heparin. Glycobiology 3, 271–277.

Manning, G.S., 1978. The molecular theory of polyelectrolyte solutions with applications to the electrostatic properties of polynucleotides. Q. Rev. Biophys. 11, 179–246.

Mardberg, K., Trybala, E., Glorioso, J.C., Bergström, 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.

Mardberg, K., Trybala, E., Tufaro, F., Bergström, T., 2002. Herpes simplex virus type 1 glycoprotein C is necessary for efficient infection of chondroitin sulfate-expressing gro2C cells. J. Gen. Virol. 83, 291–300.

McCormick, C., Leduc, L., Martindale, D., Mattison, K., Esford, L.E., Dyer, A.P., Tufaro, F., 1998. The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. Nat. Genet. 19, 158–161.

Montgomery, R.I., Warner, M.S., Lum, B.J., Spear, P.G., 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87, 427–436.

Neyts, J., De Clercq, E., 1995. Effect of polyanionic compounds on intracutaneous and intravaginal herpesvirus infection in mice: impact on the search for vaginal microbicides with anti-HIV activity. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 10, 8–12.

Oyan, A.M., Dolter, K.E., Langeland, N., Goins, W.F., Glorioso, J.C., Haarr, L., Crumpacker, C.S., 1993. Resistance of herpes simplex virus type 2 to neomycin maps to the N-terminal portion of glycoprotein C. J. Virol. 67, 2434–2441.

Record, M.T., Anderson, C.F., Lohman, T.M., 1978. Thermodynamic analysis of ion effects on the binding and conformational equilibria of proteins and nucleic acids: the roles of ion association or release screening and ion effects on water activity. Q. Rev. Biophys. 11, 103–178.

Rostand, K.S., Esko, J.D., 1997. Microbial adherence to and invasion through proteoglycans. Infect. Immun. 65, 1–8.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N.W., Bai, X., Esko, J.D., Cohen, G.H., Eisenberg, R.J., Rosenberg, R.D., Spear, P.G., 1999. A novel role for 3-*O*-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99, 13–22.

Trybala, E., Bergström, T., Svennerholm, B., Jeansson, S., Glorioso, J.C., Olofsson, S., 1994. Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulfate. J. Gen. Virol. 75, 743–752.

Trybala, E., Liljeqvist, J.-A., Svennerholm, B., Bergström, T., 2000. Herpes simplex virus types 1 and 2 differ in their interaction with heparan sulfate. J. Virol. 74, 9106–9114.

Vaheri, A., 1964. Heparin and related polyionic substances as virus inhibitors. Acta Pathol. Microbiol. Scand. 18 (Suppl. 171), 1–98.

Verrecchio, A., Germann, M.W., Schick, B.P., Kung, B., Twardowski, T., San Antonio, J.D., 2000. Design of peptides with high affinities for heparin and endothelial cell proteoglycans. J. Biol. Chem. 275, 7701–7707.

WuDunn, D., Spear, P.G., 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63, 52–58.

Zacharopoulos, V.R., Phillips, D.M., 1997. Vaginal formulations of carrageenan protect mice from herpes simplex virus infection. Clin. Diagn. Lab. Immunol. 4, 465–468.

Zsak, L., Mettenleiter, T.C., Sugg, N., Ben-Porat, T., 1990. Effect of polylysine on the early stages of infection of wild type pseudorabies virus and of mutants defective in gIII. Virology 179, 330–338.