Localization of a functional site on herpes simplex virus type 1 glycoprotein C involved in binding to cell surface heparan sulphate

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The amino acid residues critical for interaction between herpes simplex virus type 1 (HSV-1) glycoprotein C (gC-1) and cell surface heparan sulphate (HS) were localized to two separate regions within antigenic site II of this glycoprotein. These amino acids were Arg-143, Arg-145, Arg-147 and Thr-150 in one region and Gly-247 in the other. This conclusion is based on the following observations. (i) Monoclonal antibodies defining gC-1 antigenic site II, and not those reactive with antigenic site I, inhibited HSV-1-induced haemagglutination and virus binding to susceptible cells. (ii) A number of HSV-1 *mar* mutants, altered at these critical residues, were impaired in attachment to cells. (iii) Synthetic peptides,

corresponding to these two regions inhibited virus attachment to cells and infectivity. In addition these peptides were found to agglutinate red blood cells. This agglutination was inhibited by soluble HS, and was prevented by the pretreatment of red blood cells with heparitinase suggesting that cell surface HS was a site of peptide binding. The same was observed with the polycationic substances neomycin and poly-L-lysine. In conclusion, we propose that the regions of gC-1 represented by the HS-binding peptides may form a functional site of a polycationic nature, active in attachment to the polyanionic glycosaminoglycan chain of cell surface HS.

Introduction

Attachment of a virus to specific cellular receptors is the first step in the cell entry process and one of the major determinants of cell and tissue tropism. Heparan sulphate (HS) proteoglycan, a ubiquitous and multifunctional constituent of cell plasma membranes and extracellular matrices has been identified as a receptor for herpes simplex virus types 1 and 2 (HSV-1 and -2) (WuDunn & Spear, 1989; Shieh *et al.*, 1992). Of at least 11 envelope glycoproteins encoded by HSV-1 (Roizman & Sears, 1990; Hutchinson *et al.*, 1992*a, b*; Baines & Roizman, 1993), two were identified as heparin-binding proteins: glycoproteins C (gC) and B (gB) (Herold *et al.*, 1991). Although gC is regarded as the principal virus attachment protein (Herold *et al.*, 1991; Svennerholm *et al.*, 1991), the nature of the initial binding of HSV-1 to cells is largely unknown and, for at least three reasons, intriguing. First, gC-negative (gC⁻) mutants of HSV-1 show a reduced but still significant level of attachment to cells (Langeland *et al.*, 1990; Herold *et al.*, 1991; Svennerholm *et al.*, 1991) indicating that the functions of gC-1 in attachment can be replaced by other glycoprotein(s), most probably by gB (Herold *et al.*, 1991). Second, the chemically unrelated polycationic substances

neomycin and poly-L-lysine are known to interfere with HSV attachment (Langeland *et al.*, 1987, 1988, 1990) but the precise target site of their action has not yet been identified. Third, monoclonal antibodies (MAbs) and polyclonal anti-gC antibodies exhibited only moderate or low attachment inhibition activity, similar to that of antibodies to gD (Fuller & Spear, 1985), a glycoprotein involved in a step of HSV attachment/penetration subsequent to that mediated by gC (Fuller & Lee, 1992).

The possibility that initial interaction between HSV-1 and HS can be mediated by more than one viral glycoprotein (Herold *et al.*, 1991) has complicated studies on their role in attachment. One consequence of this is that little is known about the structural determinants of gC-1 involved in binding to HS. We have circumvented this difficulty by developing a haemagglutination (HA) assay, dependent on binding of gC-1 to red blood cell (RBC) surface HS (Trybala *et al.*, 1990, 1993). Here we describe localization of a functional attachment determinant of HSV-1 gC by testing (i) HA and the cell-binding activities of mutant viruses carrying defined mutations in the gC-1 gene and (ii) the inhibition of these activities by synthetic peptides and a panel of MAbs reactive with specific regions of gC-1.

Table 1. *Characteristics of mar mutants used in HSV-1 attachment study**

mar mutant	Residue no.	Amino acid change	Resistance to MABs
Mutants in antigenic site I			
C2.1	373	Pro to Gln	C1, C2, C4
C11.1	307	Gln to Arg	C1, C2, C4, C11, C14, C15
C15.1	312	Gly to Asp	C15
Mutants in antigenic site II			
C3.1	176	Glu to Lys	C16, C3
C9.1	147	Arg to Trp	C8, C9, C17, C13
C17.3	151	Arg to His	C8, C9, C17
C13.1	143	Arg to Gln	C13
C7.1	147	Arg to Trp	C3, C8, C9, C13, C7, C10
C10.1	247	Gly to Asp	C16, C3, C8, C9, C17, C13, C7, C10
C10.3	145	Arg to Gln	C3, C8, C9, C17, C13, C7, C10
C13.2	145	Arg to Gln	C8, C9, C17, C13, C7, C10
2762/B1C1	150	Thr to Ile	B1C1

* Data concerning amino acid substitutions in KOS 321 *mar* mutants and their pattern of resistance to neutralization by MABs are from Wu *et al.* (1990) and Marlin *et al.* (1985) respectively. Data concerning *mar* mutant 2762/B1C1 are from Bergström *et al.* (1993).

Methods

Cells, viruses and MABs. African green monkey kidney (GMK-AH1) cells were grown in Eagle's MEM supplemented with 2% calf serum, 0.05% Primaton RT substance, 100 U of penicillin per ml and 100 mg of streptomycin per ml. Baby hamster kidney (BHK) cells were grown in Glasgow MEM supplemented with 8% calf serum, 8% tryptose phosphate broth, 1 mM-L-glutamine and antibiotics. The HSV-1 strains used were F (Ejercito *et al.*, 1968), 2762 (Nilheden *et al.*, 1983), KOS 321, a plaque-purified isolate of wild-type strain KOS (Holland *et al.*, 1983a) and its gC⁻ mutant designated gC⁻101 (Holland *et al.*, 1984). Antigenic variants of strain KOS 321, resistant to neutralization by a single anti-gC-1 MAB (*mar* mutants), were those isolated and characterized by Marlin *et al.* (1985). The isolation of *mar* mutant 2762/B1C1 was described previously (Bergström *et al.*, 1992). The patterns of resistance of *mar* mutants to MABs (Marlin *et al.*, 1985) as well as the sites of mutation in gC (Wu *et al.*, 1990; Bergström *et al.*, 1993) are summarized in Table 1.

Of the MABs with specificity for gC-1, those designated C1 to C13 were characterized in detail elsewhere (Marlin *et al.*, 1985) whereas B1C1, C2H12 and B1E6 (an anti-gE MAB) were produced as described previously (Olofsson *et al.*, 1983). High IgG concentrations of MAB B1C1 and C2H12 were obtained by growing the hybridoma cell line in dialysis tubing (Sjögren-Jansson & Jeansson, 1985). MAB preparations were treated with acid-washed kaolin before use in an HA inhibition (HI) assay. For cell culture experiments IgG was purified from MAB samples by affinity chromatography on Protein G-Sepharose (Pharmacia). Bound antibody was eluted with a reverse flow of glycine-HCl pH 2.8, and immediately neutralized with Tris-HCl pH 8.0. Purified antibodies were centrifuged in a 10K centrifugal microconcentrator (Filtron) to concentrate IgG and to replace most of the elution buffer with PBS. The immunoglobulin concentration was determined by a standard immunodiffusion technique (Tago-Difugen kit). The reactivity of a particular MAB with KOS 321 and F strains was checked by ELISA as follows. Confluent monolayers of GMK-AH1 cells in 96-well plates were infected with HSV-1 and when the c.p.e. was advanced, the cells were fixed with 0.25% glutaraldehyde in PBS for 5 min at room temperature. The test was further carried out as previously described (Holland *et al.*, 1983b), except that *o*-phenylenediamine, dissolved in citrate buffer pH 4.5, was used as the substrate. Each MAB was tested at an IgG concentration of 10 µg/ml, and the results are presented as the difference between A_{492} values obtained in virus-infected and non-infected cells.

Preparation of radiolabelled virus. Radiolabelled, extracellular virus was separated by the modification of a method described previously (Svennerholm *et al.*, 1980). Briefly, GMK-AH1 cells in roller bottles were infected with strain KOS 321 at an m.o.i. of 3 p.f.u. per cell and the virus was allowed to adsorb for 3 h at 37 °C. The cells were then washed twice with Eagle's medium and incubated in 30 ml of fresh Eagle's medium supplemented with [³H]thymidine (40 µCi/ml) for a further 48 h at 37 °C. Culture fluid containing extracellular virus (approximately 10⁸ p.f.u./ml; HA titre 1:256) was then clarified by low-speed centrifugation and centrifuged at 15000 *g* for 5 min. The supernatant (10 ml portions) was run through a Sepharose CL-2B (Pharmacia) column (125 mm length and 25 mm inner diameter) equilibrated with Hanks' balanced salt solution, and the 5 ml fractions were tested for radioactivity, infectivity and HA. The first peak of radioactivity (9×10^5 to 1×10^6 c.p.m./ml) overlapped with peaks of virus infectivity (approximately 6×10^7 p.f.u./ml) and HA activity (titres 1:64 to 1:128). The separated virus material was supplemented with 1% BSA and stored at -70 °C. When necessary the virus was concentrated by using a centrifugal microconcentrator with a 300K cutoff.

HA and HI assays. Both assays were carried out as described previously (Trybala *et al.*, 1993). MAB samples to be used in the HI test were adjusted with the HA diluent to contain 1 mg of IgG per ml and then mixed with an equal volume of 25% suspension of acid-washed kaolin to remove non-specific inhibitors of HA. Following 30 min of agitation at room temperature, the kaolin was sedimented by centrifugation, and the supernatant (now diluted 1:2) was used in the HI test.

Attachment inhibition assay. Inhibition of HSV-1 attachment by synthetic peptides or polycations was tested as follows. GMK-AH1 cells in 24-well plates were washed with PBS and then pretreated with PBS containing 1% BSA (PBS-BSA) for 1 h at room temperature. The medium was then aspirated and 0.2 ml PBS-BSA containing indicated concentrations of peptide was added. Following incubation for 30 min at 4 °C, 20 µl of radiolabelled strain KOS 321 was added and after moderate shaking the plates were kept for adsorption in a stationary position for a further 2 h at 4 °C. The cells were then washed three times with PBS, lysed with 0.2 ml of PBS containing 5% SDS, and finally transferred to scintillation vials.

Inhibition of HSV-1 attachment by MABs was assayed by the method of Fuller & Spear (1985). Briefly, GMK-AH1 or BHK cells in 24-well plates were washed with PBS supplemented with 1% heat-

Table 2. Amino acid sequences of synthetic peptides corresponding to selected regions of HSV-1 gC

Peptide	Sequence	Amino acid no.	Purity (%)*
SP-1	GSRVQIRCFRNSTR	137-151	97
SP-1X	TRQFSRVNRSRCIRG	Scrambled SP-1	79
SP-3	PGPVWCDDRDPLARY	122-136	82
SP-4	GMYLAWGRMDSPHE	240-254	76
SP-4C	GMYTLAWGRMDSPHE	Modified SP-4	93
SP-5	LALVLPRTITMEFG	366-380	85

* The residual impurities were mainly chain-length variants of peptides.

inactivated fetal calf serum (FCS), 0.1% glucose (PBS-GC) and 0.5% BSA, and incubated with 1 ml of the same medium for 15 min at 4 °C. Five-hundred and sixty µl of affinity-purified MAb, adjusted with PBS-GC-BSA to contain 250 µg of IgG per ml, was mixed with 60 µl of radiolabelled KOS 321 (3.6×10^6 p.f.u.) and incubated for 1 h at room temperature. Portions of the mixture (150 µl) were then added in duplicate to GMK-AH1 or BHK cells and kept for attachment for 2 h at 4 °C. The rest of the procedure was as described for the assay of inhibition of HSV-1 attachment by synthetic peptides.

Purification of gC-1. gC-1 was purified from the extract of BHK cells infected with the F strain of HSV-1 by immunoaffinity (MAb B1C1) chromatography as previously described (Olofsson *et al.*, 1983).

Peptide synthesis. Based on the sequence data for gC of HSV-1 (Frink *et al.*, 1983) four 15-mer peptides corresponding to selected regions of antigenic sites I and II (Table 2) were synthesized by using Fmoc synthesis protocol (Chiron Mimotopes). Each peptide contained a free amino group and amide group at its N and C terminus respectively. Peptides were dissolved in milli-Q water and the stocks (500 µM) were stored at -20 °C. The SP-4 peptide, showing incomplete solubility in water, was dissolved in 0.1% acetic acid, and the pH was adjusted with NaOH to 7.4 when the stock was diluted further for experiments.

Interactions of synthetic peptides and polycations with surface of RBCs. Binding of synthetic peptides, neomycin (Sigma) and poly-L-lysine (M_r 3960; Sigma) to the RBC surface was tested as follows. Serial twofold dilutions of the compound in 50 µl of the HA diluent medium in 96-well, U-bottomed plates were mixed with an equal volume of a 0.4% suspension of RBCs. The type of agglutination was observed following RBC sedimentation. The same experiment was repeated with HS (preparation C, 1.5 sulphate groups per disaccharide unit) included in the HA diluent medium (10 µg per ml) or with RBCs pretreated with 2 U of heparitinase (Sigma). HS was kindly provided by Ulf Lindahl (Uppsala University, Sweden). Heparitinase-treated RBCs were prepared as previously described (Trybala *et al.*, 1993).

Results

Mapping of a functional gC-1 attachment site with MAbs

A panel of defined anti-gC-1 MAbs were tested for their ability to inhibit HSV-1 HA and cell-binding activities. MAbs 1 to 3 and 4 to 10 (Table 3) define gC-1 antigenic site I (residues 307 to 373) and site II (residues 129 to 247), respectively (Marlin *et al.*, 1985; Wu *et al.*, 1990;

Sjöblom *et al.*, 1992; Bergström *et al.*, 1993). C10, C13 and B1C1 MAbs, all of which react with antigenic site II of gC-1, inhibited HA activity of the KOS 321 and F strains of HSV-1 as well as that of gC-1 purified from the F strain (Table 3). Attachment of radiolabelled virus to cells was inhibited by all site II-defining MAbs tested albeit with markedly lower efficiency in GMK-AH1 cells compared to BHK (Table 4), suggesting a higher capacity of GMK-AH1 cells to support gC-independent binding of HSV-1. MAb B1C1, the most efficient inhibitor of virus HA, was also the most effective MAb in preventing HSV-1 adsorption to both GMK-AH1 and BHK cells. Together, the inhibition of virus HA and cell-binding activities by the antigenic site II-defining MAbs indicated that the functional attachment determinant of gC-1 was located at its N-terminal half, partly or entirely within the structures of antigenic site II. All anti-gC MAbs used gave positive ELISA signals with both KOS 321 (Table 3) and F (not shown) strains indicating that any lack of the HI or attachment-inhibiting activities was not due to the inability of a particular MAb to recognize the virus.

Mapping of a functional gC-1 attachment site with mar mutants

Since MAbs specific for the antigenic site II inhibited virus binding to cells, we sought to examine whether *mar* mutants, selected by growth in the presence of these antibodies, were impaired in attachment. To this end, we studied the kinetics of attachment to BHK cells of *mar* mutant C10.1 and its wild-type parent KOS 321. gC⁻101, a gC⁻ mutant of KOS 321, was used as a control. Mutant C10.1 showed a delay in adsorption similar to that of a gC⁻ strain indicating that the gC-1 attachment functions in *mar* mutant C10.1 were impaired (Fig. 1). Little or no

Table 3. Effect of anti-gC MAbs on HA activity of F and KOS 321 strains of HSV-1 and purified gC-1

No.	MAb	Specificity	ELISA*	HI titre		
				F	KOS 321	gC-1
1	C1	Site I	0.675	< 4	< 4	< 4
2	C2	Site I	1.096	< 4	< 4	< 4
3	C11	Site I	0.847	< 4	< 4	ND†
4	C3	Site II	1.054	< 4	< 4	< 4
5	C7	Site II	0.426	< 4	< 4	< 4
6	C8	Site II	1.146	< 4	≤ 4	< 4
7	C10	Site II	1.427	256	128	512
8	C13	Site II	1.128	ND	32	32
9	B1C1	Site II	2.450	4096	4096	4096
10	C2H12	Site II	1.353	< 4	< 4	< 4
11	B1E6	Anti-gE	1.764	< 4	< 4	< 4

* The MAb reactivity with the KOS 321 strain expressed as A_{492} value at an IgG concentration of 10 µg/ml.

† ND, Not determined.

Table 4. Effect of anti-gC MAbs on attachment of radiolabelled HSV-1 to GMK-AH1 and BHK cells*

MAb	Attached virus (% of control)	
	GMK-AH1	BHK
C2	107.43 ± 4.71	107.33 ± 9.05
C11	104.17 ± 8.31	114.42 ± 10.15
C3	94.16 ± 3.97	58.42 ± 6.04
C7†	93.80 ± 2.59	71.95 ± 5.04
C10†	86.77 ± 5.02	54.27 ± 3.80
C13	88.19 ± 8.26	63.70 ± 6.30
BIC1	69.74 ± 6.25	22.69 ± 3.33

* Two separate experiments were performed in duplicate and the results are presented as the average ± s.d. The corresponding values when heparin (25 µg/ml) was used as an inhibitor of HSV-1 attachment were 7.49 ± 0.39 and 3.74 ± 0.37 for GMK-AH1 and BHK cells respectively.

† Used in the form of ascites fluid.

Fig. 1. Kinetics of HSV-1 attachment to BHK cells. BHK cells in six-well plates were washed with cold Hanks' balanced salt solution and 200 p.f.u. of KOS 321 (○), *mar* mutant C10.1 (□) or gC⁻101 (■) in 1 ml of the same medium was added. After the indicated time of virus adsorption at 4 °C, the cells were washed three times with Eagle's medium and 2 ml of a fresh Eagle's medium containing 1% fetal calf serum was added. Following 2 h of incubation at 37 °C, 2 ml of Eagle's medium supplemented with 0.6% pooled human gammaglobulin was added and the cells were further incubated at 37 °C for 36 to 48 h. The plaques were then immunostained and counted. Each point is the average of four individual determinations from two separate experiments.

difference was found among C10.1, KOS 321 and gC⁻101 viruses when attachment kinetics were studied in GMK-AH1 cells (data not shown) again suggesting the higher capacity of these cells compared to BHK ones to support gC-independent binding of HSV-1.

Having determined that *mar* gC mutants may have a defect in attachment, we then screened a panel of 12 defined *mar* mutants for HA activity. The HA antigen

Fig. 2. HA by *mar* mutants and parental strain KOS 321. Serial twofold dilutions of the extracellular HSV-1 or supernatant fluid from uninfected GMK-AH1 cells (control) were mixed with a 0.4% suspension of mouse RBCs. The last column contains RBCs and the HA diluent only. Note differences in the type of HA, i.e. in the ability of a particular *mar* mutant to cause complete HA. KOS 321, *mar* C15.1 and *mar* C13.2 caused complete HA (designated ++ in Table 5) observed as a regular net of agglutinated RBCs at the bottom of the U-plate. *Mar* mutants C10.3 and C13.1 induced incomplete HA (denoted +) with some non-agglutinated, ring-forming erythrocytes. C10.1 virus showed no HA activity (-).

Table 5. HA activity of HSV-1 *mar* mutants

Virus	Mutation site	Infectivity (p.f.u./ml)	HA titre*	Type of HA†
KOS 321	Parent	3.9×10^8	256	++
gC ⁻ 101	gC-negative	2.6×10^8	< 2	-
C2.1	I‡	3.3×10^8	128	++
C11.1	I	9.7×10^7	128-256	++
C15.1	I	1×10^8	128-256	++
C3.1	II‡	4.5×10^8	128	++
C9.1	II	6.2×10^8	128-256	++
C17.3	II	5.4×10^8	256	++
C13.1	II	7.1×10^8	64-128	+
C7.1	II	7.3×10^8	32-64	+
C10.1	II	6.9×10^8	< 2	-
C10.3	II	6.9×10^8	32-64	+
C13.2	II	5.9×10^8	128-256	++
2762	Parent	ND§	128	++
2762/BIC1	II	ND	< 2-2	+

* HA titre range from three experiments.

† For explanations see legend to Fig. 2.

‡ Denotes antigenic site I or II.

§ ND, Not determined.

was prepared from culture fluid of GMK-AH1 cells which are known to produce a high titre of extracellular HSV-1 (Trybala *et al.*, 1993). We paid attention not only to the HA titre but also to the ability of the virus to cause complete HA, which could reflect differences in the affinity of the mutants for the RBC receptor (Fig. 2, Table 5). *Mar* mutant C10.1, which had already been found to be impaired in adsorption to BHK cells at a degree comparable to a gC⁻ strain, showed no HA. Four additional viruses carrying mutations in antigenic site II, i.e. C10.3, C7.1, C13.1 and 2762/BIC1, caused in-

complete agglutination of RBCs irrespective of the virus concentration, observed as some non-agglutinated, ring-forming cells (Fig. 2, Table 5). All other mutants exhibited complete HA at a titre comparable to, or two- to fourfold lower than the parental KOS 321 strain. The infectious titres of *mar* mutants were far above the threshold value required for HA to occur (Trybala *et al.*, 1993) indicating that the slight, or absence of, HA activity exhibited by *mar* mutants C7.1, C10.1, C10.3 and C13.1 was not caused by low concentration of the virus.

An examination of amino acid substitutions (Table 1) in *mar* mutants with impaired HA/attachment activities or none, revealed the following. (i) Mutations disrupting the attachment site of gC-1 were localized to two separate segments of this glycoprotein corresponding to amino acids 143 to 150 (C7.1, C10.3, C13.1, B1C1) and residue 247 (C10.1). (ii) The nature of these mutations was substitution of neutral amino acids for positively charged arginine residues (C7.1, C10.3, C13.1), replacement of glycine by a negatively charged aspartate residue (C10.1) or a substitution of Ile for Thr at a potential *N*-glycosylation site at residue 150 (2762/B1C1).

Effect of synthetic peptides on HSV-1 infectivity and adsorption to cells

If the above regions predicted with MABs and *mar* mutants really are critical for HSV-1 attachment, synthetic peptides corresponding to these segments of gC-1 should interfere with HSV-1 attachment to and infection of permissive cells. With this rationale we synthesized several 15-mer peptides covering the predicted attachment domain(s) on gC-1 (Table 2). Peptide SP-1 represented an arginine-rich core of gC-1 antigenic site II encompassing amino acids 143 to 150, and SP-4 spanned another gC-1 segment with possible involvement in attachment, i.e. the site of the *mar* mutation C10.1. SP-3, the peptide corresponding to the residues on the left flank of SP-1 was selected because these two segments of gC-1 contain internal Cys-127 and Cys-144 residues which according to Kikuchi *et al.* (1984) are likely to be linked in the gC molecule. Peptide SP-1X, a scrambled version of SP-1, SP-4C, a modified SP-4 with a substitution of Thr for Tyr, and SP-5, a peptide covering the site of the *mar* C2.1 mutation in antigenic site I of gC-1, were used as controls. In addition, since part of the predicted attachment determinant contained an arginine-rich segment, the polycationic substances neomycin and poly-L-lysine were included in some experiments for comparative purposes.

Peptides covering the predicted attachment determinant of gC-1, i.e. SP-1 and SP-4, were inhibitory to

Fig. 3. Effect of synthetic peptides on HSV-1 infectivity. Confluent monolayers of GMK-AH1 cells in six-well plates were washed twice with Eagle's medium and 1 ml of the same medium containing the indicated concentration of SP-1 (●), SP-1X (○), SP-3 (▽), SP-4 (▼), SP-4C (□) or SP-5 (■) peptide was added. Following 15 min of incubation at 37 °C, approximately 200 p.f.u. of the F strain was added, and after shaking the plates were kept for adsorption in a stationary position for 1 h at 37 °C. The cells were then washed three times with Eagle's medium, and a standard methylcellulose medium was added. Each point represents a mean of three replicates and the s.d. values were within 15% of the average.

HSV-1 infection of GMK-AH1 cells (Fig. 3). Peptide SP-4 exhibited a moderate effect only affecting virus infectivity by less than 50%. Peptide SP-1 was more potent and its concentration reducing the number of plaques by 50% was 22 µM. A similar effect was observed with three further samples of peptide SP-1 produced by using two different synthesis protocols (data not shown). SP-1X, a scrambled analogue of SP-1, did not inhibit HSV-1 infectivity. Peptide SP-1 showed no inhibition of virus infectivity when added 1 h after infection of GMK-AH1 cells with HSV-1 (data not shown) suggesting that the observed inhibitory effect was due to peptide interference with early events of HSV-cell interaction. To verify this, we tested the effect of the synthetic peptides on HSV-1 attachment to GMK-AH1 cells. As in the infectivity experiment, peptides were added to GMK-AH1 cells before addition of HSV-1 and were kept during the 2 h period of virus adsorption (Fig. 4a). To verify how tight the peptide interaction was with the cell surface, some cell monolayers containing pre-adsorbed peptide were washed with PBS before addition of the virus (Fig. 4b). Like polycationic substances, peptide SP-1 inhibited HSV-1 attachment to GMK cells and its concentration necessary to reduce virus attachment by 50% was 8.7 µM. The SP-1X peptide, which did not inhibit HSV-1 infectivity, reduced virus at-

Fig. 4. Effect of synthetic peptides on attachment of radiolabelled HSV-1 to cells. GMK-AH1 cells with preadsorbed SP-1 (■), SP-1X (□), SP-3 (△), SP-5 (▼), poly-L-lysine (▲) or neomycin (◇) were either not washed (a) or washed three times with PBS (b), before addition of radiolabelled virus. Each point represents a mean of three replicates and the s.d. values were within 11% of the average. The concentration of neomycin was 200-fold higher than indicated on the bottom of the figure.

tachment but only at the highest concentration tested (50 µM). This can be attributed to the different conditions under which attachment and infectivity assays were carried out (note that SP-1 was more active in the attachment assay than in the infectivity assay, by over twofold). SP-4 also inhibited virus binding to cells. This peptide was tested at a concentration of only 50 µM, and the inhibitory effect expressed as a percentage of virus attachment in the absence of peptide was 40.62 ± 6.27 and 93.56 ± 4.60 when the GMK-AH1 cells with preadsorbed SP-4 were washed before addition of virus. The corresponding values for the control peptide, SP-4C,

Fig. 5. Agglutination of mouse RBCs by neomycin, poly-L-lysine and synthetic peptides. Serial twofold dilutions of the indicated compound were mixed with a 0.4% suspension of mouse RBCs (row A). The same experiment was repeated with HS included in the HA diluent medium (row B) or with the use of heparinase-treated RBCs (row C). The concentrations of neomycin were twofold higher than indicated at the bottom of the figure.

were 108.44 ± 1.78 and 104.68 ± 11.86 respectively (data not shown). When GMK-AH1 cells, pretreated with peptides or polycations, were washed with PBS before addition of the virus, no inhibiting compounds other than SP-1 and poly-L-lysine maintained the inhibition of HSV attachment to cells (Fig. 4b).

Peptides SP-1 and SP-4 were also tested for their ability to inhibit HA by HSV-1 strains KOS 321 and F. SP-1 inhibited viral HA from a concentration of 0.1 μM , whereas the effect of SP-4 could not be examined owing to agglutination of RBCs induced by this peptide (data not shown).

Binding of the blocking peptides to cell surface HS

Having determined that synthetic peptides representing the predicted attachment determinant of gC-1 inhibited virus infectivity, attachment and HA activity, we attempted to define whether these effects were due to peptide-HS interaction. For this purpose we tested the binding of the peptide to the RBC surface both in the presence of soluble HS and after the treatment of RBCs with heparitinase. Similar approaches, based on experiments performed in cell cultures, were used to identify HS as a receptor for HSV (WuDunn & Spear, 1989). We observed that SP-1, SP-4 and polycationic substances caused incomplete agglutination of mouse RBCs (Fig. 5, rows A). Neomycin and SP-4 induced similar agglutination of RBCs at concentrations above 1.6 μg per ml. In contrast poly-L-lysine caused HA in the range 0.1 to 1.6 μg per ml and SP-1 exhibited weak agglutination at 0.05 to 0.1 μg per ml with classical prozone effects at higher concentrations. The evidence for the prozone effect was that HSV-1-induced HA was inhibited by both SP-1 and poly-L-lysine in the concentration range at which the prozone was observed (data not shown). In contrast, peptides SP-1X and SP-3 caused RBC agglutination at much higher concentrations whereas SP-5 and SP-4C exhibited no RBC agglutination. RBC agglutination induced by SP-1, SP-4, poly-L-lysine and neomycin was not found when HS was included in the diluent medium (10 μg per ml) or when mouse RBCs were pretreated with heparitinase (Fig. 5, rows B and C) indicating that HS was a site of binding of peptides from the predicted gC-1 attachment determinant as well as a site of binding of polycationic substances neomycin and poly-L-lysine.

Discussion

Using a panel of anti-gC MAbs, *mar* viruses carrying specific mutations in the gC gene and synthetic peptides we attempted to localize a functional site on HSV-1 gC-1 involved in binding to cell surface HS. The summary of our results is presented in Fig. 6. The amino acid residues critical for gC-mediated attachment of HSV-1 to HS were localized to two separate regions of this glycoprotein, one including Arg-143, Arg-145, Arg-147 and Thr-150, and the other containing Gly-247. Since mutation at Thr-150 was found to be associated with the absence of *N*-linked glycan at position 148 (Bergström *et*

al., 1993) this component seemed also to be involved in the formation of the functional attachment site.

Although the two regions engaged in HS binding are separated on the primary gC-1 sequence by almost 100 amino acids, both are included in the epitope of MAb

Fig. 6. (a) Schematic structure of HSV-1 gC showing the location of amino acid residues critical for virus binding to HS and the positions of the epitopes for MAbs that were the most potent in the inhibition of virus attachment to cells (open boxes). The positions of antigenic sites I and II are indicated. The lollipops and the hatched box show the positions of *N*- and *O*-linked glycans respectively. (b) Polypeptide stretches of HSV-1 gC involved in virus binding to cells. The positions of the residues critical for gC-1 attachment function are indicated by numbers printed in boldface. The *N*-linked glycan at position 148 is depicted, the silhouette mimics the van der Waal surface of a diantennary complex-type *N*-linked glycan and its size is adjusted to correspond to the distance between adjacent amino acids in the peptide linkage. The disulphide bonding is according to the report by Kikuchi *et al.* (1984).

C10 (Wu *et al.*, 1990), and therefore are likely to be located in close proximity in the three-dimensional structure of glycoprotein, forming a single attachment determinant. In addition, residues adjacent to these two regions were reported to contribute to the binding of the C3b component of complement further emphasizing the association between these two stretches in the native gC-1 (Hung *et al.*, 1992).

A striking feature of the suggested attachment site is that it is of polycationic nature since part of it contains an arginine-rich segment. In accordance, polycationic substances such as neomycin, poly-L-lysine and poly-arginine are known to compete with HSV attachment to the cells (Langeland *et al.*, 1987, 1988, 1990; WuDunn & Spear, 1989; Campadelli-Fiume *et al.*, 1990). Our observation that these substances can bind to cell surface HS suggested that gC-HS interaction relies to a large extent on ionic interaction. One important question is how simple ionic interactions may account for a high degree of specificity usually associated with the binding between the virus attachment protein and its receptor. Synthetic peptide SP-1 comprising the arginine-rich segment bound specifically and irreversibly to cell surface HS. SP-1X, a control peptide with the same net positive charge as SP-1, was inactive or exhibited significantly lower activity in the attachment assays further emphasizing the high specificity of interaction between SP-1 and HS. In contrast, SP-4 bound reversibly to HS whereas synthetic peptides corresponding to other regions of gC-1 bound weakly or not at all to HS even if some of them contained, like SP-1X, basic amino acid residues. This showed that the presence of basic amino acids per se is not sufficient to generate an irreversible, high affinity binding to HS; the topological distribution of such amino acids must be a critical parameter.

Since the HS glycosaminoglycan chain is built up of repetitive units it would be expected that the binding motif should be a regular structure of high order. Indeed, arginine residues in the irreversibly binding peptide are arranged in this way, forming a motif 137-RxxxRCRFR-xxxR-151 with three critical arginines in the centre separated by hydrophobic amino acids. In our preliminary studies we found that the substitution of either histidine or threonine for F-146 drastically reduced the affinity of SP-1 for HS (E. Trybala, unpublished results) suggesting that the proper arrangement of arginines and hydrophobic residues might be necessary for specific spatial interaction with the negatively charged sulphate esters or carboxylic acid groups on the glycosaminoglycan chain of HS. This is in line with recent reports where consensus sequences, containing clusters of basic amino acids, have been proposed as heparin-binding determinants for a number of biologically active ligands (for review see Jackson *et al.*, 1991). In addition,

comparison of the gC-1 amino acid sequence with its bovine herpesvirus type 1 (BHV-1) and pseudorabies virus (PRV) counterparts, gIII, revealed the existence of similar peptide stretches 177-RVGRFR-182 and 136-RRGRFR-141 for BHV-1 (Fitzpatrick *et al.*, 1989) and PRV (Robbins *et al.*, 1986) respectively with phenylalanine at a similar location.

By using PRV mutants with defined deletions in the gIII gene, Flynn *et al.* (1993) demonstrated that the functional attachment site of that molecule was located at its one-third N-terminal portion (amino acids 25 to 157). On the other hand, Liang *et al.* (1993), using a panel of partially overlapping BHV-1 and PRV gIII peptides, identified multiple heparin-binding sites on these molecules. The majority of these heparin-binding peptides inhibited neither virus attachment to cells nor infectivity, and the only peptide effective with PRV (Liang *et al.*, 1993) corresponded to the gIII segment located 100 amino acids downstream from the region predicted to contain the attachment site (Flynn *et al.*, 1993). This indicates that the ability of a particular peptide to interact with heparin does not necessarily imply that it has a biological function. Indeed, some residual activity associated with our control peptide SP-1X indicates that there may be some level of non-specific peptide-HS interactions. In addition, it should be taken into account that HS is one of the strongest polyanions in the mammalian organism and may interact in a more or less specific manner with a variety of polycationic substances.

The function of the other part of the HS-binding site of gC-1 encompassing Gly-247 seems to be more complex. On the one hand, SP-4, a synthetic peptide representing this region, bound to HS with significantly lower affinity than SP-1, and consequently gave only a moderate inhibition of HSV-1 attachment and infectivity. In fact, this peptide contained only two basic amino acids, i.e. arginine and histidine, located in an environment of aspartate and glutamate. SP-4, however, comprised several aromatic residues including tryptophan which was recently proposed to be required for binding of some peptides from the human thrombospondin type I repeats to heparin (Guo *et al.*, 1992). On the other hand, the C10.1 mutant with the *mar* phenotype defined by a substitution of Asp for Gly-247 completely lacked the ability to bind to HS via gC-1 which is in sharp contrast to the weaker effect of mutations in the arginine-rich region. It is reasonable to assume that it was not an elimination of Gly-247 per se, but the introduction of an aspartate residue into its position, which was responsible for the lack of HS-binding activity in HSV-1 *mar* mutant C10.1. As mentioned above, gC-1 polypeptide stretches comprising Gly-247 and the Arg-143 to Arg-147 segment are likely

to be structurally, antigenically and functionally connected. Consequently the negative charge of the Asp-247 side-chain may abolish the affinity of arginine residues for HS. It is also noteworthy that this substitution of Asp for Gly has a profound effect on antigenic properties of *mar* mutant C10.1 since this virus lost reactivity with all MAbs defining antigenic site II of gC-1 (Marlin *et al.*, 1985).

Apart from the attachment function, gC-1 acts also as a receptor for the C3b component of complement (Friedman *et al.*, 1984). It is likely that the functional domains responsible for these two binding activities are not identical. First, binding of complement was inhibited by MAbs specific for both antigenic sites I and II (Friedman *et al.*, 1986), whereas a pattern of HI and attachment inhibition was restricted to the MAbs defining site II. Second, expression of C3b-binding activity by particular *mar* gC mutants (Friedman *et al.*, 1986) differed from their HA activity presented in this paper. Finally, of the four regions identified as important for C3b-binding (Hung *et al.*, 1992) only segment IV, located between residues 223 and 246, partly overlapped with our HS-binding SP-4 peptide from the site of the *mar* C10.1 mutation. In contrast, SP-3, corresponding to residues 122 to 136, i.e. a gC-1 segment that was almost identical with region I contributing to C3b binding (Hung *et al.*, 1992), had little or no HS-binding ability.

Finally, although we cannot rule out a possibility that some other regions of gC-1 may contribute to the formation of a functional HSV-1 attachment site, the results of our experiments with MAbs, *mar* mutants, and synthetic peptides indicate that the two regions of gC-1 form a functional domain involved in binding to cell surface HS. Owing to the polycationic nature of this site, it appears that HSV-1 may mimic the way in which other biologically important ligands interact with HS. We are currently examining variants of the parent peptide SP-1 to localize the minimum sequence sufficient for HS binding, and to understand the nature of the irreversible interaction between this peptide and HS.

We thank Maria Johansson for excellent technical assistance, Gerd Wolff for the preparation of cell cultures and Nancy Nenonen for correcting the English and for many helpful suggestions. This work was supported by grants from the Swedish Medical Research Council (Grants 9083, 9483 and 10437), the National Swedish Board for Technical Development (Project 87-0256P), the Medical Faculty, University of Göteborg, a grant to J.C.G. from NIH (Grant GM34534), and the Hartmann Foundation, Copenhagen. E.T. was supported by a grant from the Swedish Institute.

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(Received 6 September 1993; Accepted 4 November 1993)