

Cell Surface Receptors for Herpes Simplex Virus Are Heparan Sulfate Proteoglycans

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Abstract. The role of cell surface heparan sulfate in herpes simplex virus (HSV) infection was investigated using CHO cell mutants defective in various aspects of glycosaminoglycan synthesis. Binding of radiolabeled virus to the cells and infection were assessed in mutant and wild-type cells. Virus bound efficiently to wild-type cells and initiated an abortive infection in which immediate-early or α viral genes were expressed, despite limited production of late viral pro-

teins and progeny virus. Binding of virus to heparan sulfate-deficient mutant cells was severely impaired and mutant cells were resistant to HSV infection. Intermediate levels of binding and infection were observed for a CHO cell mutant that produced undersulfated heparan sulfate. These results show that heparan sulfate moieties of cell surface proteoglycans serve as receptors for HSV.

IDENTIFICATION of a particular cell surface molecule as a receptor for a virus, or identification of multiple cell surface molecules as alternative receptors, must be based on evidence satisfying at least two criteria. First, presence of the receptor (or one of multiple receptors) should be prerequisite for binding of virus to a cell and for subsequent infection. Conversely, absence of the putative receptor(s) should prevent specific binding of the virus to the cell and should render the cell resistant to infection. Second, it should be possible to show that the virus actually interacts physically with the cell surface molecule(s) in question. The purpose of this paper is to report genetic evidence that the cell surface glycosaminoglycan (GAG)¹, heparan sulfate, fulfills these criteria for identification as a receptor for herpes simplex virus (HSV).

HSV is isolated principally from humans under natural conditions although the virus has a broad host range under experimental conditions. A variety of laboratory animals can be infected with the virus and most adherent vertebrate cells grown in culture are susceptible to HSV infection. This implies that the cell surface receptors for HSV are ubiquitous and highly conserved or that different receptors may be used for the infection of different cell types.

Two kinds of cell surface molecules have been implicated as receptors for HSV: heparan sulfate moieties of cell surface

proteoglycans (67) and a high affinity receptor for basic FGF (29).

Two lines of evidence suggested that heparan sulfate serves as receptor for HSV. First, removal of heparan sulfate from human cells, by enzymatic treatment, reduces the ability of the cells to bind virus and renders the cells at least partially resistant to HSV infection (67). Second, the viral glycoprotein designated gC is principally responsible for the binding of HSV to cells and has affinity for heparin (22), which is closely related in structure to heparan sulfate (30).

Two lines of evidence also suggested initially that FGF receptors have a role in HSV adsorption to cells or in entry. First, basic FGF was shown to inhibit HSV binding to cells and HSV infection (29). Second, it appeared that greater quantities of labeled virus could bind to CHO cells transfected to express a receptor for FGF than to receptor-deficient CHO cells (29), although this result could not be reproduced (55).

The idea that the binding of HSV to cells might require interaction with heparan sulfate as well as with some other cell surface molecule (29, 67) is important to consider, in light of other findings. For example, the high-affinity binding of basic FGF to cells and subsequent biological responses require the dual interaction of basic FGF with cell surface heparan sulfate (or soluble heparin) and other protein receptors (48, 68). In addition, the participation of neuronal cell adhesion molecule (N-CAM) in neuronal cell-cell and cell-substratum adhesions depends upon interactions of N-CAM with heparin-like molecules (10, 11). Also, fibronectin has specific binding domains both for cell surface heparan sulfate and for receptors of the integrin superfamily (50). Although the adhesion of cells to substrata depends principally on integrin-fibronectin interactions, the reorganization of cyto-

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1. *Abbreviations used in this paper:* GAG, glycosaminoglycan; HEp-2, human epidermoid carcinoma No. 2; HSV, herpes simplex virus; N-CAM, neuronal cell adhesion molecule; PFU, plaque-forming units.

skeletal components that accompanies this adhesion depends on heparan sulfate–fibronectin interactions (33, 66).

Here we have made use of CHO cell mutants defective in various aspects of GAG synthesis to test further the roles of cell surface heparan sulfate and FGF receptors in HSV infection. Most adherent vertebrate cells produce a diverse array of proteoglycans (21, 23). The three principal GAGs found on cell surface proteoglycans are heparan sulfate, chondroitin sulfate, and dermatan sulfate. Heparan sulfate is chemically very similar to commercial heparin, except that heparin tends to be the more highly sulfated polymer and is produced by specialized types of cells (30). A variety of mutant CHO cell lines isolated by Esko and colleagues (14, 15) have been shown to have specific defects that interfere with various aspects of GAG biosynthesis (Table I). CHO mutants in complementation groups pgsA and pgsB fail to produce GAGs because they are deficient in one of the enzymes required to form the common tetrasaccharide that is linked to the protein core and upon which all three types of GAG are then polymerized. CHO mutants in complementation groups pgsD and pgsE have defects affecting the production of heparan sulfate but not other GAGs. Specifically, pgsD mutants lack enzymatic activities required to form the repeating disaccharide unit characteristic of heparan sulfate and pgsE mutants produce undersulfated heparan sulfate because of a deficiency of the enzyme required to add sulfate to deacetylated amino groups in the glucosamine moieties (a type of sulfation that is not found in the other GAGs present on cell surfaces). Both the wild-type and mutant CHO cells used in this study have been reported to be deficient in the high affinity receptors for FGF (68).

Our results show that presence of heparan sulfate on cell surfaces, but not other GAGs, is the principal requirement for the binding of HSV to cells and for subsequent infection of the cells. Moreover, the level of N-sulfation of heparan sulfate significantly influences the amount of virus that can bind to and infect the cells. Results presented here, coupled with other findings presented elsewhere (43, 55), also show that receptors for FGF do not have a role in HSV infectivity, despite previous reports proposing this hypothesis (1, 29). It is possible, for reasons to be discussed, that cell surface receptors in addition to heparan sulfate are important for HSV penetration and infectivity. However, these other (hypothetical) receptors remain to be identified.

Materials and Methods

Cells and Viruses

Wild-type CHO-K1 cells and the GAG-deficient mutants have been described previously (see citations in Table I). The cells were passaged in Ham's F12 medium supplemented with 10% FBS. Human epidermoid carcinoma No. 2 (HEp-2) cells were passaged in DME supplemented with 10% FBS. The viruses used were HSV-1(KOS), HSV-1(F), and HSV-2(333), and the Indiana strain of vesicular stomatitis virus. Titrations of plaque-forming units (PFU) were done on HEp-2 cells for HSV (22) and on the cells indicated for vesicular stomatitis virus.

ICP4 Expression as Determined by Immunofluorescence Assay

Cells were plated on 12-mm coverslips in 5-ml glass scintillation vials (13-mm diam; Research Products International Corp., Mount Prospect, IL) and incubated at 37°C overnight. The cells were inoculated with HSV in PBS

(10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, 2.5 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂) supplemented with 0.1% glucose and incubated for 6 h at 37°C (the first 2 h of incubation were on a shaker). The inoculum volume was 0.1 ml and the virus concentrations were adjusted to achieve the desired input multiplicity of infection (PFU/cell). The cells were then treated with a periodate-lysine-paraformaldehyde fixative (39) and permeabilized with 0.1% Triton X-100 in PBS. Hybridoma culture fluid containing an anti-ICP4 mAb (either 58S [reference 56] or H640-28 [reference 25]) was diluted 1:2 with PBS containing 1% BSA (PBS-BSA) and incubated with the cells for 30 min at room temperature. The cells were washed with PBS three times to remove unbound antibodies, and then incubated with a 1:100 dilution of FITC-conjugated F(ab)₂ fragments of goat anti-mouse IgG antibodies (Organon Teknika Corp., Cappel Research Products, Durham, NC) for 30 min at room temperature. After washing, the coverslips were mounted in medium (5% propyl gallate, 60% glycerol in PBS) for examination. For each sample, two counts of ~250 cells in different fields were made to quantitate the numbers of ICP4-positive and ICP4-negative cells. The values given are the averages of two counts, expressed as percent of cells that were ICP4-positive. Error bars in figures indicate the range of values obtained for the duplicate samples.

Binding of Radiolabeled HSV to Cells

HSV virions were labeled and purified as described (8, 57). Briefly, roller bottles of HEp-2 cells were inoculated with virus at 3–5 PFU/cell for 2 h. After removal of inoculum, the cells were overlaid with medium 199 supplemented with 1% FBS, with or without [³⁵S]methionine (15 μCi/ml) or [³H]thymidine (20 μCi/ml) and incubated at 37°C for 48–72 h. When the infected cells were labeled with [³⁵S]methionine, the medium 199 used had 20% of the normal levels of cold methionine. Virus was harvested and purified from infected-cell cytoplasmic lysates by centrifugation through dextran gradients (Dextran T10; Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Infectious virus was quantitated by titrations on HEp-2 cells and radioactivity was quantitated by liquid scintillation counting. Particle numbers were determined by EM (22, 42). Most virus preparations had titers of 10⁸ to 10⁹ PFU/ml. Particle/PFU ratios were usually ~50:1 for HSV-1 (KOS) and HSV-2(333), and about 2–5:1 for HSV-1(F). Just before use, virus was diluted 1:4 (vol/vol) in PBS, centrifuged at 40,000 g for 1 h, and resuspended in PBS.

For the binding experiments the cells were plated either in 96-well round-bottomed plates (Gibco BRL, Gaithersburg, MD), 24-well plates, or on the bottoms of the glass scintillation vials described above, and incubated at 37°C overnight. The confluent monolayers were pretreated with PBS-BSA for 1 h at 37°C. The cells were then washed three times with PBS-BSA and inoculated with purified labeled virus in a sufficient volume of PBS-BSA to keep the cells covered with fluid. The cells were exposed to the labeled virus for 6 h at 4°C on a shaker (pilot experiments revealed that 6 h was required for the binding of virus to approach equilibrium) and the cells were then washed three times with cold PBS. If the cells had been plated on 96-well plates, they were transferred to scintillation vials after detachment with EDTA and scintillation fluid was added. If the cells had been plated in the scintillation vials, then scintillation fluid was simply added for the quantitation of radioactivity bound to the cells. The values given are the averages of duplicate samples. The results are expressed as number of virions bound per cell, based on the specific activities of the virus preparations (virions per cpm). Error bars in figures indicate the range of values obtained.

Cell Killing Assay

Cells growing in 25-cm² flasks were inoculated with virus in PBS containing 0.1% glucose. After 6 h of incubation at 37°C, the cells were detached with trypsin-EDTA. The detached cells were then replated in 75-cm² flasks containing growth medium, at ~1,000 cells per flask. After 10 d incubation, the colonies were fixed and stained with Giemsa for quantitation.

Results

Wild-Type CHO Cells Are Susceptible to HSV Infection

To assess the biological significance of the binding of virus to cells, it is important to quantitate infection in parallel with binding. Because CHO cells were previously reported to be

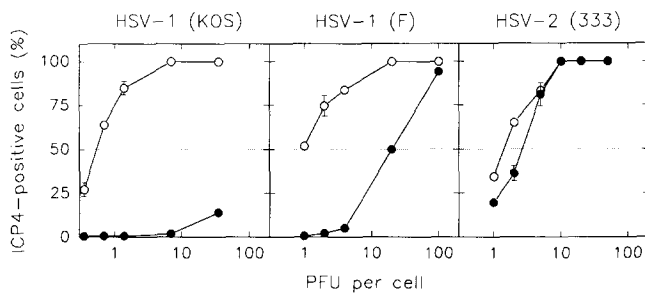


Figure 1. Expression of an immediately-early HSV protein (ICP4) after infection of HEP-2 cells and wild-type CHO cells with HSV-1 (KOS), HSV-1(F), or HSV-2(333). HEP-2 cells (○) or CHO cells (●) were exposed to various concentrations of virus for 6 h at 37°C. The cells were then fixed and stained for detection of the viral protein ICP4 by immunofluorescence. The percentage of cells with fluorescent nuclei was determined. The values given for multiplicity of infection (PFU per cell) are based on titrations of PFU on HEP-2 cells.

nonpermissive for HSV replication (24), we tested whether wild-type CHO cells are susceptible to HSV infection nonetheless. A cell was judged to be successfully infected if it expressed one of the immediate-early or α viral proteins, indicating that adsorption, penetration, and uncoating of the viral genome had occurred and that viral gene expression had commenced. Expression of the α viral protein designated ICP4 was assessed by immunofluorescence using a mAb specific for this viral regulatory protein. Wild-type CHO cells and human HEP-2 cells (fully permissive for HSV replication) were exposed to equivalent doses of each of three different HSV strains and then processed for immunofluorescence at 6 h after addition of the virus.

Fig. 1 shows that all three strains of HSV induced ICP4 expression in the wild-type CHO cells but at considerably different efficiencies compared with the ability of each strain to induce ICP4 expression in HEP-2 cells. The ED₅₀ (dose of virus required to induce ICP4 expression in 50% of cells) was similar for HSV-2(333) on both the wild-type CHO cells and HEP-2 cells. In contrast, the ED₅₀ for HSV-1(F) on CHO cells was more than 10 times that observed on HEP-2 cells. This differential was even greater for HSV-1(KOS). We conclude that the wild-type CHO cells are fully susceptible to infection by some strains of HSV (such as HSV-2[333]) but that some factors present in, or absent from, the CHO cells restrict infection by the other strains of HSV tested. This restriction is partial and is probably not at the level of the binding of virus to cells, based on data presented in the next section. It seems likely, therefore, that the bottleneck for infection by some strains of HSV is at the level of viral penetration of the cell, uncoating of the genome, or expression of viral genes.

Expression of late viral proteins in infected CHO cells was below normal levels and few if any infectious progeny virions were produced (data not shown). It seems likely that CHO cells do not provide the factors required for optimal expression of many HSV genes. Alternatively, CHO cells may express inhibitory factors that block expression of intermediate or late viral genes.

One feature of HSV infection in permissive cells is that soluble heparin concomitantly blocks the binding of virus to the cells and infection of the cells (22, 67). Fig. 2 shows that

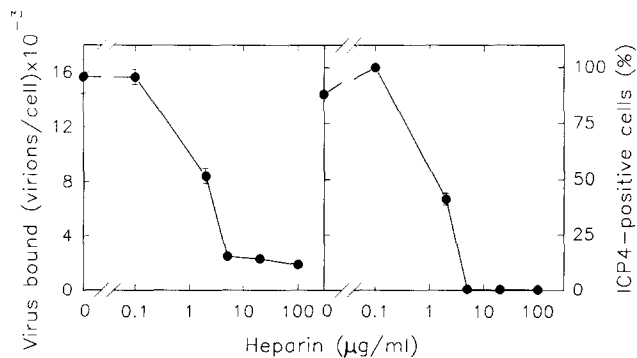


Figure 2. Effect of heparin on HSV binding and ICP4 expression in wild-type CHO cells. The cells were plated in scintillation vials with and without coverslips. Samples of purified HSV-1(KOS) (³H]thymidine-labeled virus at 8.7×10^4 virions/cpm) were mixed with various concentrations of heparin immediately before inoculation of the cells. The cell number was 1.23×10^5 per vial. Virus added was 5.6×10^9 virions per vial or 4.5×10^4 virions per cell. Cells in vials without coverslips were exposed to virus for 6 h at 4°C. Unbound virus was then washed away and cell-bound radioactivity was quantitated. Cells in vials with coverslips were exposed to virus for 6 h at 37°C, for the quantitation of ICP4 expression as described in the text and legend to Fig. 1.

heparin inhibits both virus binding to and infection of wild-type CHO cells as well. In this experiment the cells were exposed to purified labeled HSV-1(KOS) at a high input dose (900 PFU/cell), in the absence or presence of heparin at the concentrations indicated, and then replicate samples were processed either for quantitation of virus binding to the cells or for quantitation of ICP4 expression by immunofluorescence. Several points should be made about the results. First, HSV-1(KOS) is capable of inducing ICP4 expression in 100% of CHO cells, provided the infecting dose of virus is large enough (compare Figs. 1 and 2). Second, the inhibition of ICP4 expression by heparin can be fully explained by the inhibition of the binding of virus to the cells. We assume that the residual binding of label to the cells at the highest concentrations of heparin is nonspecific inasmuch as ICP4 expression was completely inhibited at these concentrations.

It should be noted that CHO cells are markedly deficient in high affinity receptors for FGF (38, 68) and yet are susceptible to HSV infection. Enhancement of FGF receptor expression by transfection of the appropriate gene into CHO cells does not enhance susceptibility of the cells to infection by HSV-1(F) or HSV-1(KOS) (55). Thus, deficiency of FGF receptors does not account for the partial restriction of HSV infection observed for these HSV strains in control CHO cells. In other studies we are exploring the molecular basis for the variability in efficiency with which different HSV strains infect CHO cells. The focus of this study, however, is to test the effects of CHO mutations that impair GAG biosynthesis on expression of HSV receptors and on susceptibility of the cells to HSV infection.

CHO Mutants with Defects in Heparan Sulfate Biosynthesis Are Resistant to HSV Infection and Have Reduced Numbers of Receptors for HSV

The inhibition of HSV infection by heparin and by treatment of cells with heparitinase (67) and the affinity of specific

Table 1. CHO Cell Lines Used and Their Properties

Cell type	Strain	Biochemical deficiency	GAGs produced		Reference
			Heparan sulfate	Chondroitin sulfate	
Wild-type	K1	None	Yes	Yes	
Mutant group					
pgsA	745	Xylosyltransferase	No	No	(15)
pgsB	761	Galactosyltransferase	No	No	(16)
pgsD	677	<i>N</i> -acetylglucosaminyl and glucuronosyltransferases	No	Yes*	(17) (34)
pgsE	606	<i>N</i> -sulfotransferase	Yes†	Yes	(2) (3)

* Chondroitin sulfate accumulates to levels three times higher than in wild-type cells.

† The heparan sulfate produced is undersulfated by a factor of two to three.

HSV glycoproteins for heparin (22) suggested that cell surface heparan sulfate may serve as receptor for HSV. To test this possibility, the susceptibility to HSV infection of wild-type CHO cells and the GAG-deficient mutants (Table I) was compared (Fig. 3). Each of the cell lines indicated was exposed to a range of concentrations of HSV-1(KOS), HSV-1(F), or HSV-2(333) and infection was monitored by quantitating the number of cells expressing ICP4. With all three strains of virus, the wild-type cells were most susceptible to infection and the heparan sulfate-deficient mutant cells were least susceptible, regardless of whether the mutant cells expressed undetectable or elevated levels of chondroitin sulfate (Table I). The mutant cells (pgsE-606) that produce undersulfated heparan sulfate had an intermediate level of susceptibility to HSV infection. The difference in susceptibility of the wild-type and heparan sulfate-deficient cells was very pronounced. For example, both pgsA-745 and pgsD-677 cells were almost totally resistant to concentrations of HSV-1(F) capable of infecting 100% of wild-type cells. Although a small percentage of the pgsA-745 and pgsD-677 cells could be infected by HSV-2(333), much higher input doses of virus were required than was required to infect a comparable percentage of wild-type cells. The altered susceptibility of cells

caused by defects in heparan sulfate biosynthesis were superimposed on the differences in ability of the three viral strains to infect CHO cells (Fig. 1).

The binding of HSV to mutant and wild-type CHO cells was also assessed. Monolayer cultures of the different cell lines were exposed to a range of concentrations of purified radiolabeled virus at 4°C and then washed to quantitate the amount of virus that remained bound to the cells. The results presented in Figs. 4 and 5 show that there was very little, if any, specific (heparin-inhibitable) binding to the heparan sulfate-deficient mutants regardless of whether the mutants produced chondroitin sulfate. The low levels of binding were usually slightly higher for the chondroitin sulfate-producing, heparan sulfate-deficient mutant (pgsD-677) than for the other mutants, but this did not correlate with proportionately greater ICP4 expression (Fig. 3). HSV-2(333) reproducibly exhibited more heparin-inhibitable binding to the heparan sulfate-deficient cells than did the other two strains of virus. This correlated with somewhat greater, but still low, expression of ICP4 by HSV-2(333) than by the other virus strains tested. All three strains of virus exhibited intermediate levels of binding to the mutant cells that produced under-

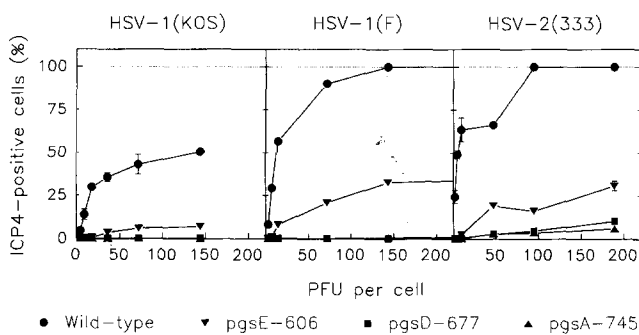


Figure 3. Expression of ICP4 after infection of wild-type CHO cells and GAG-deficient CHO mutants with HSV-1(KOS), HSV-1(F), or HSV-2(333). Wild-type and mutant cells were plated in scintillation vials with coverslips and then exposed to various concentrations of the virus strains indicated. ICP4 expression was quantitated at 6 h after infection as described in the legend to Fig. 1. The virus input is based on the virus titer on HEp-2 cells. The highest dose tested for HSV-1(F) (290 PFU/cell) is not included on the graph; the values obtained at 290 PFU/cell were not significantly different from the values obtained at 145 PFU/cell.

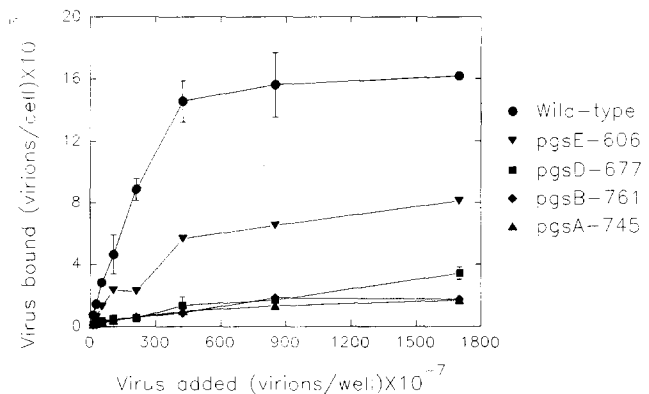


Figure 4. Binding of HSV-1(KOS) to wild-type CHO cells and GAG-deficient CHO mutants. Wild-type and mutant cells were plated in 96-well round-bottomed plates, and inoculated with various concentrations of purified [³⁵S]methionine-labeled HSV-1(KOS) (3.35×10^4 virions/cpm). For the different cell lines, the cell numbers ranged from 2.7 to 5.8×10^4 per well. The adsorption period was 6 h at 4°C. After unbound virus was washed away, the cells were detached with EDTA and transferred to scintillation vials for the quantitation of cell-bound radioactivity.

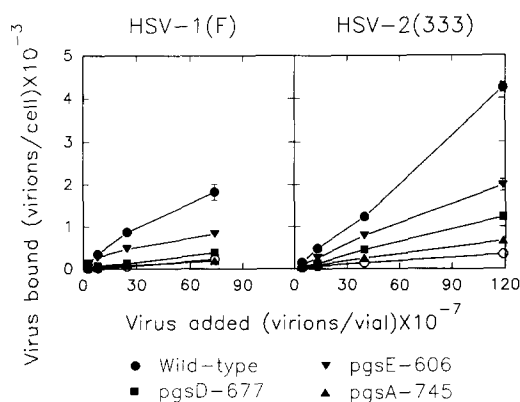


Figure 5. Binding of HSV to wild-type CHO cells and GAG-deficient CHO mutants in the presence (O) or absence (●, ■, ▲, ▼) of heparin. Wild-type and mutant cells were plated in scintillation vials and inoculated with purified, [³H]thymidine-labeled HSV-1(F) or HSV-2(333) at different subsaturating concentrations of virus in the presence or absence of heparin (20 μg/ml). The specific activities of the virus preparations were 3.9×10^4 virions/cpm for HSV-1(F) and 5.0×10^4 virions/cpm for HSV-2(333). For the different cell lines, the cell numbers ranged from 7 to 16×10^4 per vial (HSV-1[F]) and from 8 to 15×10^4 per vial (HSV-2[333]). The adsorption period was 6 h at 4°C. Cell-bound virus was quantitated as described in the legend to Fig. 2. The level of binding in the presence of heparin was essentially the same for all cell lines; the average heparin-inhibited values for all cell lines are represented by the open circles.

sulfated heparan sulfate. Interestingly, half-maximal binding of HSV-1(KOS) was comparable for pgsE-606 and wild-type cells but the plateau levels differed by two- to threefold (Fig. 4). The diminution in HSV binding to the pgsE-606 cells correlates well with the decrease in overall sulfation of heparan sulfate (3). The results presented in Figs. 3–5 indicate that the resistance of the mutant CHO cells to HSV infection can be accounted for by the reduced levels of binding of HSV to the cells.

We have not detected differences in binding of the different HSV strains to wild-type CHO cells that could account for the different efficiencies with which each strain induces ICP4 expression in the wild-type cells (Figs. 1 and 5). Thus the polymorphic viral genes that determine the efficiency of ICP4 induction in CHO cells must influence some step between binding of virus to the cell and early viral gene expression.

Yayon et al. (68) used some of the CHO cell mutants employed in this study to transfect a gene for an FGF receptor and to show that the binding of basic FGF to this receptor requires the presence of cell surface heparan sulfate. Basic FGF could bind to the transfected receptor on heparan sulfate-deficient cells only if heparin was added at low concentrations. Because basic FGF is a heparin-binding protein, the authors hypothesized that interaction of basic FGF with exogenous heparin or cellular heparan sulfate alters the conformation of basic FGF, which enables its binding to the high affinity receptor. An experiment was done to determine whether low concentrations of exogenous heparin could enhance the binding of HSV to heparan sulfate-deficient cells (Fig. 6). Heparin ranging in concentration from 1 ng/ml to inhibitory levels failed to enhance the binding of HSV to heparan sulfate-deficient cells. These concentrations of hep-

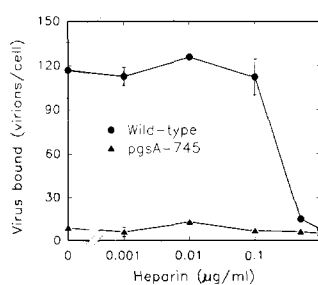


Figure 6. Effect of low-dose heparin on binding of HSV to wild-type and heparan sulfate-negative mutant CHO cells. Wild-type and pgsA-745 cells were plated in 24-well plates containing coverslips and exposed to purified [³⁵S]methionine-labeled HSV-1(KOS) (2.4×10^4 virions/cpm) mixed with heparin at the concentrations indicated. Cell numbers were 4×10^5 per well. Virus added was 1.6×10^9 virions per well. The adsorption period was for 6 h at 4°C. After washing away unbound virus, the coverslips were transferred to scintillation vials for the quantitation of cell-bound radioactivity. The relatively low efficiency of virus binding to wild-type cells in the absence of heparin was because of the larger volume of fluid used for virus adsorption (0.3 ml rather than 0.1 ml) and resultant lower concentration of the added virus.

arin also failed to enhance infection of the heparan sulfate-deficient cells, as assessed by testing for ICP4 expression (results not shown). The failure of exogenous heparin to enhance binding of HSV suggests that the virus requires cell-bound heparan sulfate, presumably in the form of membrane proteoglycan.

Because abortive infections with HSV can be cytotoxic, we examined the survival of wild-type CHO cells and mutants after challenge with virus. Quantitative results were obtained using a colony-forming assay. Wild-type and mutant cells were exposed to various doses of virus. After 6 h, the cells were detached and replaced at low density and cell colonies were counted 10 d later. Consistent with the results presented in Fig. 1, HSV-2(333) killed wild-type CHO cells more efficiently than did HSV-1(F), which in turn killed these cells more efficiently than did HSV-1(KOS) (data not shown). For comparison of the susceptibility of wild-type and mutant cells to killing by HSV-2(333), the cells were exposed to 15 or 150 PFU per cell of virus (Fig. 7). At the highest dose of virus used, the wild-type CHO cells were essentially all killed whereas all of the mutants were resistant to killing by HSV-2(333). It is interesting that even the mutant cell line producing undersulfated heparan sulfate was completely resistant to killing. At the highest dose of HSV-2(333) used, ~25% of the cells should have been infected, as judged by the results of other similar experiments (Fig. 3). Therefore it seems likely that killing of CHO cells requires entry of multiple virions. The possibility exists that the virion-associated factor responsible for the immediate shut-off of host protein synthesis (18, 49) contributes to the cytotoxicity of HSV-2(333). Consistent with this possibility, it has been shown that HSV-2 expresses a much more potent shut-off factor than HSV-1 (19) and that full activity requires entry into the cell of multiple virions (31).

Mutant and Wild-type CHO Cells Are Equally Susceptible to Infection by Vesicular Stomatitis Virus

The heparan sulfate-deficient CHO cells are not resistant to infection by all viruses. The results presented in Table II show that vesicular stomatitis virus formed plaques with equal efficiency on wild-type and mutant CHO cells. Four

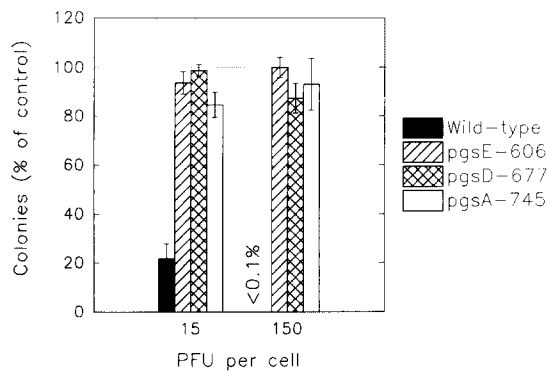


Figure 7. Viability (colony-forming ability) of wild-type and GAG-deficient mutant CHO cells after exposure to HSV-2(333). Wild-type and mutant cells were plated in 25-cm² flasks and exposed to various concentrations of HSV-2(333). After 6 h of incubation at 37°C, the cells were detached and replated at low density. After 10 d incubation, the colonies were stained and counted. The number of colonies for each sample is expressed as the percentage of colonies obtained from mock-infected cells. The values shown are the averages of duplicate determinations and error bars represent the range of values obtained.

virus stocks, prepared on BHK cells or on wild-type CHO cells, had similar titers of PFUs on all three CHO cell lines tested. In contrast, the titers of HSV ICP4-inducing units were so much lower on the heparan sulfate-deficient mutants than on wild-type CHO cells that accurate quantitation of the differences could not be done.

Discussion

The results presented here provide genetic evidence that heparan sulfate serves as cell surface receptor for HSV and is required as receptor for the usual pathway by which this virus attaches to and invades a cell. We show that absence of heparan sulfate renders CHO cells deficient for HSV receptors and resistant to HSV infection. This conclusion is generalizable to primate cells. Although primate cell mutants defective for heparan sulfate synthesis have not been described, use of heparitinase or heparinase to selectively remove heparan sulfate from human or monkey cells has qualitatively the same effect on HSV binding and infection (67) as the CHO cell mutations described here. Because

Table II. Plating Efficiencies of Vesicular Stomatitis Virus on Wild-Type CHO Cells and Mutants

Virus stock*	10 ⁸ plaque-forming units [‡]		
	Wild type	pgsA-745	pgsB-761
VSV-BHK	2.9	4.3	4.9
VSV-CHO-K1	2.5	3.0	1.1
VSV-CHO-K1	3.0	4.8	3.1
VSV-CHO-K1	6.5	8.5	6.0

* Virus stocks were prepared on BHK cells or on CHO-K1 cells as indicated. The cells were infected at 0.01 PFU/cell and incubated for 2 d in Ham's F12 medium containing 2% FBS. The medium was cleared of cell debris and used as the source of virus for the titrations.

‡ Dilutions of each virus stock were plated on wild-type CHO-K1 cells, pgsA-745 cells, or pgsB-761 cells as indicated. The cells were incubated for 2 d in the medium described above containing 0.95% Noble agar. Plaques were then counted.

soluble heparin could not enhance the binding of HSV to heparan sulfate-deficient cells, it seems likely that the interaction of virus with heparin-like molecules does not serve merely to facilitate the binding of virus to some other receptor. The simplest interpretation of the findings summarized here is that cell surface heparan sulfate serves as the HSV receptor.

The heparan sulfate to which HSV binds must consist largely of heparan sulfate chains covalently attached to cell surface proteoglycans. Although free GAG chains can bind to cells, exogenous heparin could not enhance the binding of HSV to cells (Fig. 6) and most cell surface GAGs are covalently attached to a protein core (30). CHO cells, like most other cell types, express multiple cell surface proteoglycans containing heparan sulfate. Recent studies indicate that a proteoglycan related to syndecan (52) is a major cell surface proteoglycan in CHO cells (Zhang, L., and J. D. Esko, unpublished results). CHO cells also express a proteoglycan that is the type III receptor for transforming growth factor- β (TGF- β). Interestingly, the GAG chains normally present on this receptor (heparan sulfate and chondroitin sulfate) are dispensable for expression of the receptor on cell surfaces and for binding and function of TGF- β , as shown by studies done with the CHO mutants pgsA-745 and pgsB-761 (9). In contrast with TGF- β , HSV binds to heparan sulfate and probably not to the protein core, at least not in the absence of the heparan sulfate moieties of the proteoglycan. For HSV, it remains to be determined whether any heparan sulfate proteoglycan can serve as receptor or whether a particular proteoglycan is required. Specific proteoglycans may be preferred as HSV receptors because of characteristic modifications of their heparan sulfate chains. In addition, HSV virions may bind to diverse heparan sulfate proteoglycans but be activated for penetration of the cell only after interaction with particular proteoglycans, due perhaps to interactions of the virus with molecular determinants of both heparan sulfate and the protein core or to requirement for proteoglycans that are physically linked to other cell components involved in viral penetration.

Characterization of the virion glycoprotein that mediates the binding of HSV to cells supports the conclusion that heparan sulfate is the receptor. Herold et al. (22) showed that two of the virion glycoproteins (gB and gC) have heparin-binding activity and that gC is principally responsible for the binding of virions to cells. This latter conclusion was based on finding that gC-negative virions are impaired in ability to bind to cells whereas absence of any of the other glycoproteins known to be required for HSV infectivity (gB, gD, and gH) blocks penetration of virus into cells but not binding of virus to cells (5, 12, 35, 36, 51). Studies defining the molecular target of an inhibitor of HSV infection also indicate that gC has a role in the binding of virus to cells (7, 32). Although gC-negative virions exhibit specific infectivities less than 1/10 that of wild-type virus, there is significant residual infectivity which also depends on the presence of cell surface heparan sulfate. We have proposed that there is some redundancy in viral functions that can mediate the binding of virus to cells and that the other heparin-binding glycoprotein (gB) may substitute for gC, albeit with lower efficiency (22).

The requirement of HSV for heparan sulfate as receptor cannot be met by the other GAGs commonly found on cell surfaces, such as chondroitin sulfate. The evidence includes

the finding that CHO cell mutants capable of expressing chondroitin sulfate, but not heparan sulfate, are almost as resistant to HSV binding and infection as CHO cell mutants that fail to produce any GAGs. In addition, treatment of primate cells with enzymes capable of selectively removing chondroitin sulfate and dermatan sulfate has no effect on susceptibility of the cells to HSV binding and infection (67). These results imply that virion glycoproteins recognize structural features of heparan sulfate that are not shared with other GAGs.

The repeating disaccharide unit of heparan sulfate consists of the amino sugar *N*-acetyl- or *N*-sulfo-glucosamine and a hexuronic acid whereas the repeating disaccharide unit of chondroitin sulfate and dermatan sulfate consists of *N*-acetylgalactosamine and a hexuronic acid (23). Possibly, the amino sugar is an important determinant in specificity of the HSV antireceptor. It is perhaps relevant in this regard that the binding of HSV to cells can be inhibited by pretreatment of the cells with wheat germ agglutinin (65, 69), which can bind to *N*-acetylglucosamine residues. Degree of sulfation of the heparan sulfate is also clearly an important determinant of specificity in the interaction of HSV with cells, given the reduced number of HSV receptors on pgsE-606 cells, which produce undersulfated heparan sulfate. Consistent with these findings, it was recently shown that binding of HSV to cells is inhibited most effectively by the more highly sulfated fractions of heparan sulfate or heparin and very poorly, if at all, by chondroitin sulfate or dermatan sulfate (37).

It is clear from the results presented here and previously (67) that cell surface heparan sulfate is required for the binding of both serotypes of HSV (HSV-1 and HSV-2) to cells. Because the HSV-1 and HSV-2 forms of gC and gB differ in amino acid sequence (4, 13, 58, 59), the possibility exists that there are serotype-specific differences in the structural features of heparan sulfate required for virus binding. Such differences could explain previous findings that the relative binding of HSV-1 and HSV-2 to different cell types is not constant (62, 63). The precise requirements for interactions of the various forms of HSV gB and gC with heparin or heparan sulfate remain to be defined.

After the binding of HSV to a cell, penetration of virus into the cell can occur by fusion of the virion envelope with the cell plasma membrane (20, 44, 47, 65). The events required for viral penetration are not yet known but depend upon the activities of at least three HSV glycoproteins designated gB, gD, and gH (5, 12, 35, 36, 51). The possibility exists that one or more of these glycoproteins may interact with a cell surface receptor distinct from heparan sulfate. If any of these glycoproteins engages in interactions with receptors other than heparan sulfate, however, these interactions must usually occur secondary to the binding of virus to heparan sulfate and must not be of high enough affinity to enable efficient binding of virus to cells in the absence of heparan sulfate. It has been proposed that gD may bind to a specific cell surface component (6, 26–28). Absence of gD from virions, however, does not impair binding of the virions to cells; gD-negative virions are noninfectious due to improvement of penetration (35). Thus, gD may interact with cell surface components involved in fusion of the viral envelope with the plasma membrane.

Recently it was claimed that high affinity receptors for FGF might serve as "portals of entry" into cells for HSV

(29). This conclusion rested on findings that basic FGF could inhibit HSV infection of endothelial cells and that labeled material in virus preparations bound to a greater extent to CHO cells transfected with an FGF receptor than to control CHO cells. Subsequent studies have shown that this enhanced binding could not be reproduced by the authors or us and that the FGF receptor-positive CHO cells and control cells were indistinguishable in susceptibility to HSV infection (55). In addition, Mirza et al. (43) showed that FGF receptor-positive and FGF receptor-negative rat myoblasts did not differ in susceptibility to HSV infection and that a soluble form of the FGF receptor failed to inhibit HSV infection whereas it did inhibit basic FGF binding and biological activity. The most likely explanations for the original results reported by Hajjar and collaborators (1, 29) are competition between HSV and basic FGF for binding to cell surface heparan sulfate (43, 55) and presence of contaminating material (including basic FGF) in their labeled virus preparations (55). It has been shown that other heparin-binding proteins (such as platelet factor 4) can block both the binding of HSV to cells and infection of the cells (67).

Viral receptors for HSV and for at least two of the animal herpesviruses — pseudorabies virus and bovine herpesvirus type 1 — appear to be similar or related. Evidence that heparan sulfate also serves as receptor for these animal viruses comes from findings that heparin inhibits infection and that treatment of cells with heparitinase or heparinase reduces the binding of virus to the cells and renders the cells at least partially resistant to virus infection (41, 46, 53). In addition, both of these animal viruses encode glycoproteins that are related to HSV gC and, for both viruses, the gC homolog has heparin-binding activity and is principally responsible for the binding of virus to cells (40, 41, 46, 53, 54, 64, 70). These similarities among HSV, pseudorabies virus, and bovine herpesvirus type 1 are consistent with the similarities in biological properties and pathogenesis of the viruses (despite their propensity to infect different animal species).

With any virus, it is necessary to consider whether there is a single pathway for viral binding and entry into cells or multiple pathways. It seems likely that the high efficiency infection of most cultured adherent cells with HSV depends upon the presence of cell surface heparan sulfate. This assertion is based on findings that heparin inhibits infection by HSV of many cell types and species (45, 60, 61, 67) and that absence of cell surface heparan sulfate renders human, monkey, and rodent cells resistant to HSV infection (67). On the other hand, the heparan sulfate-deficient CHO cells could be infected, albeit inefficiently, by HSV-2(333). This suggests that the mutant cells may express low levels of heparin-like molecules, that some other cell surface molecule can substitute poorly for heparan sulfate, or that some alternate, but inefficient, pathway exists for the functional binding of HSV to cells.

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