Initial Interaction of Herpes Simplex Virus with Cells Is Binding to Heparan Sulfate

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We have shown that cell surface heparan sulfate serves as the initial receptor for both serotypes of herpes simplex virus (HSV). We found that virions could bind to heparin, a related glycosaminoglycan, and that heparin blocked virus adsorption. Agents known to bind to cell surface heparan sulfate blocked viral adsorption and infection. Enzymatic digestion of cell surface heparan sulfate but not of dermatan sulfate or chondroitin sulfate concomitantly reduced the binding of virus to the cells and rendered the cells resistant to infection. Although cell surface heparan sulfate was required for infection by HSV types 1 and 2, the two serotypes may bind to heparan sulfate with different affinities or may recognize different structural features of heparan sulfate. Consistent with their broad host ranges, the two HSV serotypes use as primary receptors ubiquitous cell surface components known to participate in interactions with the extracellular matrix and with other cell surfaces.

Recent work has confirmed earlier proposals about the pathway of herpes simplex virus (HSV) entry into a cell. Following attachment of the virion to the cell surface, the viral envelope fuses with the plasma membrane (16, 30, 33) and the viral capsid is released into the cytoplasm. The adsorption and penetration events probably require at least three of the seven envelope glycoproteins encoded by the viral genome (36). Genetic evidence demonstrates that the glycoproteins designated gB, gD, and gH are required for virus entry into a cell, at least in part for steps occurring after the initial attachment of virions to the cells (3, 4, 11, 25, 26, 35). Moreover, neutralizing monoclonal antibodies specific for each of these three glycoproteins can block viral penetration with little or no effect on virion adsorption to cells (16, 19, 20; A. O. Fuller, R. Santos, and P. G. Spear, manuscript in preparation). The other four known glycoproteins are apparently dispensable for viral replication in cultured cells (12, 21, 27, 32). That gD and gB are present in different morphologically distinct spikes projecting from the virion envelope (38) implies a requirement for multiple virion-cell surface interactions subsequent to adsorption in order for virion-cell fusion to occur. Cell surface molecules necessary for the initiation of HSV infection have not been identified until now.

Glycosaminoglycans are long, polyanionic carbohydrate chains consisting of repeating disaccharide units. In proteoglycans, these polymers are covalently attached to a protein core. The glycosaminoglycan heparin has long been known to inhibit the infection of cultured cells by HSV but not by many other viruses (31, 39, 40). The basis for this inhibition was not understood. Because heparan sulfate proteoglycans, which carry glycans similar to heparin, are present on the surface of most types of vertebrate cells, we explored the possibility that heparan sulfate serves as a receptor for HSV. We conclude that both HSV types 1 and 2 (HSV-1 and HSV-2) normally initiate infection by binding first to cell surface heparan sulfate. The probable necessity for subsequent receptor interactions at the cell surface is discussed.

MATERIALS AND METHODS

Viruses and cells. HEp-2 cells were obtained from the American Type Culture Collection. Cells were passaged in Dulbecco modified Eagle (DME) medium supplemented with 10% fetal bovine serum. HSV-1(KOS) was kindly provided by Priscilla Schaffer of the Harvard Medical School. HSV-2(G) has been described previously (13), and Sendai/RU virus was kindly provided by Robert Lamb of Northwestern University.

Virus purification. Virions purified as described by Spear and Roizman (37) and modified by Cassai et al. (6) were used for all experiments. Briefly, roller bottles of HEp-2 cells were inoculated with virus at 3 PFU/cell for 2 h. Following removal of the inoculum, cells were overlaid with growth medium, with or without [3H]thymidine (20 μCi/ml) or [35S]methionine (15 μCi/ml), and incubated at 37°C for 48 h. Virus was harvested and purified from infected-cell lysates by centrifugation through dextran gradients (Dextran T10; Pharmacia). Titters were determined by plaquing on HEp-2 cells, and radioactivity was determined by liquid scintillation counting. Most virus preparations had titers of 10⁸ to 10⁹ PFU/ml and radioactivities of 10⁵ to 10⁷ cpm/ml. Just prior to use, virus was diluted 1:4 (vol/vol) in phosphate-buffered saline (PBS: 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 140 mM NaCl, 2.5 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂) and centrifuged in a Centricron-30 concentrator (Amicon), according to the manufacturer's directions, to remove most of the dextran.

Inhibitory agents and enzyme digestions. Heparin (Sigma), poly-L-lysine (Sigma), and platelet factor 4 (Sigma) were dissolved at the appropriate concentrations in PBS with or without purified virus. Heparitinase, heparinase, and chondroitin ABC lyase (ICN ImmunoBiologicals) digestions were done in PBS-0.1% glucose–0.1 to 1.0 mg of bovine serum albumin per ml–100 mM phenylmethylsulfonyl fluoride or 500 U of aprotinin per ml. Amounts used are expressed in conventional units (1 conventional unit = 6 mIU) per milliliter. None of these enzyme treatments caused any detachment of cells from the monolayers.

Viral plaque assays. For viral plaque assays, untreated or enzyme-treated HEp-2 cells in 25-cm² flasks were inoculated

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with virus in PBS supplemented with 0.1% glucose and, in some instances, containing one of the inhibitory agents to be tested. After a 30-min incubation, the viral inoculum was removed and the cells were overlaid with DME containing 1% heat-inactivated fetal bovine serum and 0.1% pooled human gamma globulin. Plaques were counted after 3 to 4 days. For Sendai virus plaquing, HEp-2 cells were inoculated with virus in DME containing 1% bovine serum albumin. Following incubation for 45 min at 37°C, the viral inoculum was removed and replaced with DME supplemented with 1% bovine serum albumin, 1 μg of acetylated trypsin (Sigma) per ml, and 0.7% SeaPlaque agarose (FMC Bioproducts). Plaques were counted after 2 days.

**Binding of radiolabeled virus to cells.** Confluent monolayers of HEp-2 cells in 24-well cluster dishes were pretreated for 15 min at 37°C with PBS containing 1% fetal bovine serum, 0.1% glucose, and bovine serum albumin (5 mg/ml) in order to block nonspecific virus adsorption. This pretreatment was omitted for the enzyme-treated cells. The cells were then inoculated with radiolabeled virions (about 10^7 PFU or 10^5 cpm) in 0.15 ml of PBS supplement with bovine serum albumin (1 to 2 mg/ml) and, in some instances, containing one of the inhibitors to be tested. After 30 min of adsorption at 37°C, the virus inoculum was removed and the cells were rinsed at least three times with PBS. Cells, along with any bound or penetrated virus, were lysed in PBS–1% sodium dodecyl sulfate (SDS)–1% Triton X-100. The lysates were then dissolved in Ecolume (ICN BioMedicals), and radioactivity was counted in a scintillation counter.

**Affinity chromatography.** One-milliliter columns of heparin-Sepharose (Pharmacia), Sepharose (Pharmacia), and polylysine-agarose (Pierce) were equilibrated with PBS. Virus samples were loaded in 1 ml of PBS, and the columns were then washed with 10 ml of PBS. Material flowing through the columns from the loading and washing procedures was pooled and assayed for infectivity and radioactivity.

**Labeling of cell surface glycosaminoglycans.** To label glycosaminoglycans, HEp-2 cells were incubated for 48 h with sulfate-deficient minimal essential medium supplemented with [35S]sulfate (20 μCi/ml). Cells were rinsed six to eight times to remove residual radioactive sulfate.

**Purification and synthesis assays.** Confluent monolayers of HEp-2 cells in 24-well dishes were treated with heparitinase or heparinase at 1 U/ml for 1 h at 37°C and then washed with PBS. Following viral adsorption for 30 min at 37°C, cells were overlaid with DME medium containing 10% fetal bovine serum. At 5 h postinfection, cells were rinsed with PBS and then overlaid with medium 199 lacking methionine and supplemented with [35S]methionine (20 μCi/ml). After 30 min, the cells were rinsed with PBS and lysed in SDS sample buffer (10 mM sodium phosphate buffer [pH 7.0], 1% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.001% bromophenol blue). The lysates were boiled for 15 min and then loaded on an 8.5% polyacrylamide–SDS slab gel (N-N’-diallyltartardiamide cross-linked). Following electrophoresis, the gels were fixed, soaked in Amplify (Amersham), dried, and exposed to film at −70°C.

**Competition assay.** For competition assays, relative particle numbers in labeled and unlabeled preparations of purified HSV-1 and HSV-2 were determined by quantitating relative amounts of the VP5 (capsid) protein. Equal volumes of the various viral preparations were electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE). Following silver staining, the gels were scanned by optical densitometry. Electron microscopy confirmed that in all virus preparations, about 85% of the virus particles were enveloped. To determine the amount of virus required to saturate the cell surface, increasing concentrations of [35S]methionine-labeled HSV-1(KOS) were adsorbed to confluent monolayers of HEp-2 cells in 96-well (round-bottomed) microtiter plates for 2 h at 4°C. The cells were washed with PBS, and the amount of radioactivity remaining associated with the cells was quantitated by lysing the cells and measuring radioactivity by liquid scintillation counting. Saturation of radiolabeled virus binding occurred at 2 × 10^9 PFU/ml. In the competition assay, cells in 96-well (round-bottomed) plates were inoculated at 4°C for 2 h with a constant saturating amount of radiolabeled virus (2.2 × 10^9 PFU/ml; about 670 PFU/cell) plus various amounts of competing unlabeled virus. Following several rinses of the cells, the cells and any associated virus were lysed in PBS–1% SDS–1% Triton X-100, and the radioactivity in the lysates was counted in a liquid scintillation counter.

**RESULTS**

**Inhibitors of HSV adsorption and infection.** We tested whether early events in HSV infection, in particular adsorption, could be inhibited by agents that should block the putative attachment of virus to heparan sulfate. The potential inhibitory agents tested were heparin, poly-l-lysine, and platelet factor 4. Heparin and poly-l-lysine should bind to polyacations and polyanions, respectively, present on the surfaces of virions and cells. Platelet factor 4 is a small, slightly basic protein with specific affinity for glycosaminoglycans, especially heparin and heparan sulfate. Polybrene, a polycation, and protamine sulfate, a heparin antagonist, have previously been shown to reverse the effects of heparin as well as inhibit HSV infection when used alone (40).

These agents were incubated with HEp-2 cells either immediately before or during exposure of the cells to purified HSV-1 strain KOS [HSV-1(KOS)] or HSV-2 strain G [HSV-2(G)]. Effects on plaque formation and on adsorption of radiolabeled virions to the cells were assessed. When plaque formation was monitored, replicate cultures of cells were incubated with agent immediately after the period of virus adsorption also in order to control for postadsorption effects on plaque formation.

Figure 1 shows that heparin inhibited plaque formation, as previously reported, and also inhibited virion adsorption, but only when present during virus adsorption. Poly-l-lysine and platelet factor 4 also inhibited plaque formation and virion adsorption. In contrast to heparin, however, these agents had inhibitory effects whether they were incubated with cells before or during exposure of the cells to virus. Inhibition of plaque formation by heparin and platelet factor 4 can be explained by inhibition of virus attachment to cells, whereas the inhibition of plaque formation by poly-l-lysine is only partly attributable to effects on virus adsorption (Fig. 1).

Interestingly, while both HSV serotypes were affected by these agents, HSV-1 adsorption and infection were less sensitive to inhibition by heparin but more sensitive to inhibition by platelet factor 4 in comparison with HSV-2 (note the difference in scale for the heparin dose). Furthermore, poly-l-lysine partially inhibited HSV-1 adsorption but had no significant effect on HSV-2 adsorption despite its marked effect on both HSV-1 and HSV-2 infectivity. Clearly, for both HSV-1 and HSV-2, poly-l-lysine inhibited a postadsorption step required for the initiation of infection,
TABLE 1. Affinity chromatography of radiolabeled virions

<table>
<thead>
<tr>
<th>Affinity column</th>
<th>Unbound materiala (% of material applied)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1 (KOS)</td>
</tr>
<tr>
<td>Heparin-Sepharose CL6B</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>104</td>
</tr>
<tr>
<td>Polylysine-agarose</td>
<td>91</td>
</tr>
<tr>
<td>Sepharose CL6B</td>
<td>90</td>
</tr>
</tbody>
</table>

* The material applied to the columns contained $10^6$ to $10^7$ PFU and about $10^8$ to $10^9$ cpm.

Antagonists (2, 18), had no effect on HSV infectivity (data not shown).

**Affinity chromatography.** Affinity chromatography experiments showed that virions could bind to heparin but not to polylsine (Table 1). Binding of virions to heparin-Sepharose was virtually complete, whereas no significant binding to Sepharose or polylsine-agarose could be detected.

The binding of HSV-1 to heparin-Sepharose was blocked by preequilibration of the column with soluble heparin (2 mg/ml) and inclusion of heparin (2 mg/ml) in the elution buffer. In the presence of soluble heparin, over 90% of the input radioactivity and from 19 to 36% of input PFU were recovered. The disparity between radioactivity and PFU recovered may be due to deleterious effects on virus of long exposure to heparin. Short-term incubations with heparin had no irreversible effects on virion infectivity. In contrast, the presence of soluble heparin did not significantly increase the recovery of HSV-2 (either PFU or cpm) from heparin-Sepharose (data not shown). This suggests that, unlike HSV-1 (KOS), HSV-2 (G) has a much higher affinity for the heparin linked to Sepharose than for the soluble heparin preparation.

**Effects of enzyme treatments.** HEp-2 cells were exposed to three different enzymes capable of digesting cell surface glycosaminoglycan moieties. Heparinase cleaves glycosidic linkages present in heparin and in some heparan sulfates, while heparitinase has selectivity for linkages present in heparan sulfate. Chondroitin ABC lyase specifically cleaves glycosidic linkages present in chondroitin sulfates A and C and in dermatan sulfate (chondroitin sulfate B), the other major glycosaminoglycans found on cell surfaces. When HEp-2 cells labeled with [$^{35}$S]sulfate were incubated with each of these three enzymes, comparable amounts of radioactive material were released from the cells. The cells treated with chondroitin ABC lyase appeared normal in their ability to be infected and to support plaque formation, whereas the cells treated with heparinase or heparitinase exhibited greatly reduced numbers of plaques compared with control cells (Table 2).

We treated HEp-2 cells with various concentrations of the three enzymes to assess their effects on the ability of the cells to adsorb virus and to become infected. Cells treated with chondroitin ABC lyase were indistinguishable from control cells in both assays (data not shown). Digestion with either heparitinase or heparinase before virus adsorption, but not after, resulted in reduced numbers of plaques on the treated cells and in reduced efficiency of virion adsorption (Fig. 2). In contrast, heparitinase or heparinase pretreatment of HEp-2 cells had no effect on the infectivity of Sendai virus, which also enters cells by fusing with the plasma membrane, but after adsorption to sialic acid on specific cell surface gangliosides (7, 28). In an experiment in which heparitinase reduced the number of HSV-1 plaques to 20%...
TABLE 2. Enzymatic release of glycosaminoglycans from HEp-2 cells and effects on HSV-1 (KOS) plaque formationa

<table>
<thead>
<tr>
<th>Enzyme (U/ml)</th>
<th>35S-labeled material released (cpm/culture)</th>
<th>Plating efficiency (PFU/culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15,000</td>
<td>350</td>
</tr>
<tr>
<td>Heparinase (1)</td>
<td>68,000</td>
<td>48</td>
</tr>
<tr>
<td>Heparitinase (1)</td>
<td>64,000</td>
<td>33</td>
</tr>
<tr>
<td>Chondroitin lyase (2)</td>
<td>52,000</td>
<td>320</td>
</tr>
</tbody>
</table>

a Cell monolayers were incubated with [35S]sulfate for 48 h to label glycosaminoglycans and then rinsed with PBS until no significant radioactivity could be detected in the rinses. Following enzyme treatment for 1 h at 37°C, medium was removed for liquid scintillation counting and cells were inoculated with purified virus. The duplicate counts of cpm and PFU differed by less than 20% from the averages shown.

The effects of the enzymes on HSV infection of HEp-2 cells were clearly due to alterations of the cells and not of the virus. Not only were the cells washed free of enzyme before addition of virus, but deliberate treatment of virus with either heparitinase or heparinase did not alter the infectivity of the virus. The observed effects of enzymatic treatment of the cells were due to the specific activities of the enzymes used and not to contaminating activities. This was shown by adding increasing amounts of soluble heparan sulfate or heparin to the reaction mixtures during preadsorption enzymatic treatment of the cells. Soluble heparan sulfate at concentrations approaching 2 mg/ml could prevent the action of either heparitinase or heparinase in rendering the cells resistant to infection as assayed by plaque formation. Heparin (at 0.3 mg/ml) prevented the action of heparinase but not of heparitinase (Fig. 3).

As another test of the resistance of enzyme-treated cells to HSV infection, treated and untreated HEp-2 cells were also exposed to relatively high concentrations of virus at 37°C and then pulse-labeled at 5 h after addition of virus. SDS-PAGE profiles of labeled proteins from the infected cells showed that the amount of pulse-labeled viral proteins produced by control cells was roughly proportional to the concentration of virus used for infection over the range tested (Fig. 4). Heparinase- and heparitinase-treated cells could be infected but only at the highest concentrations of virus used. The concentration of HSV-1 applied to the enzyme-treated cells had to be 5 to 10 times higher than that applied to control cells to obtain comparable amounts of viral protein synthesis. The enzyme-treated cells were even less susceptible to HSV-2 infection. Viral protein synthesis was barely detectable, if at all, after exposure of enzymetreated cells to the highest doses of HSV-2 tested.

Competitive binding of the two serotypes. The preceding results strongly suggest that the initial interaction of both HSV-1 and HSV-2 with cells is binding to heparan sulfate. However, slight differences in the behavior of HSV-1 and HSV-2 indicate possible differences in their interactions with heparan sulfate. We performed competitive binding experiments to determine whether adsorption of one serotype would interfere with adsorption of the other. Cells were inoculated with a saturating concentration of radiolabeled HSV-1 (KOS) along with various concentrations of nonlabeled homotypic or heterotypic virus. Both serotypes were able to competitively inhibit the binding of the labeled HSV-1 to approximately the same extent (Fig. 5).

DISCUSSION

Our results demonstrate that the initial step in infection of HEp-2 cells by HSV-1 and HSV-2 is binding of virions to
heparan sulfate on the cell surface. Agents that can block this interaction effectively block HSV adsorption and infection. Heparin binds to virions, presumably occupying the sites necessary for attachment of virions to cell surface heparan sulfate. Platelet factor 4 blocks HSV adsorption and infection by modifying the cell. Presumably it binds to heparan sulfate and occupies sites to which virions normally bind. Enzymatic removal of heparan sulfate from the cell surface significantly reduced the efficiency with which the cells could be infected. This implies that heparan sulfate is either required for infectivity or greatly enhances the efficiency of infection. Although the virus selectively interacts with heparan sulfate, we cannot rule out low-efficiency interactions with other cell surface polyanions.

The results obtained with HEp-2 cells are probably generalizable to other cell types. Certainly, heparin has been tested for its ability to inhibit HSV infection of a variety of cell types, with uniformly positive results (39; unpublished results). Moreover, heparan sulfate in multiple forms is present on the surface of many cell types (22). Besides being a constituent of the extracellular matrix, the proteoglycan can be intercalated into the plasma membrane via its protein moiety, with its glycosaminoglycan chains on the outside of the cell (23, 34). Cell surface heparan sulfate is required for certain cell-cell and cell-substratum interactions (8, 9, 17, 24).

Mutant derivatives of Chinese hamster ovary (CHO) cells have been isolated and shown to be deficient in various aspects of glycosaminoglycan synthesis (14). Because the CHO cell line is one of the few cultured cell types that does not support HSV replication, it is not ideal for studies of HSV entry. Nevertheless, we have tested two mutant derivatives of the CHO cell line (kindly provided by J. Esko, University of Alabama) and found that radiolabeled HSV-1 and HSV-2 bound very poorly if at all to the mutant cells, whereas both bound efficiently to the parental wild-type CHO cells. The mutants tested lacked xylosyltransferase (CHO 745) or galactosyltransferase I (CHO 761) and were deficient in synthesis of all glycosaminoglycans (14, 15). We are currently analyzing additional mutants with more selective defects in synthesis of specific classes of glycosaminoglycans.

Previous studies indicated that prior adsorption to cells of inactivated or mutant HSV-1 could inhibit subsequent infection with HSV-1 but not with HSV-2, suggesting that the two serotypes used different cell surface receptors or different pathways of entry (1, 41, 42). The experiments reported here show that both HSV-1 and HSV-2 make their initial contact with cells through binding to heparan sulfate and that they compete with each other for this initial contact.

The changes previously observed for HSV-1 and HSV-2 entry may involve steps subsequent to initial attachment. Multiple interactions between the virion envelope and plasma membrane probably occur after attachment and prior to viral penetration by fusion of the virion envelope with the plasma membrane. The seven HSV glycoproteins, especially the three glycoproteins known to be required for HSV
HSV BINDS INITIALLY TO CELL SURFACE HEPARAN SULFATE

infectivity, may all participate in these interactions. Some of these HSV-1 and HSV-2 glycoproteins may have diverged sufficiently that the pathways of HSV-1 and HSV-2 entry diverge after the first step.

There are several compelling reasons for proposing that the interaction of HSV-1 or HSV-2 with heparan sulfate is only the first in a cascade of interactions culminating in fusion of the virion envelope with the plasma membrane. First, both gB and gD are required for HSV penetration, but not necessarily for adsorption, and each is found in a different structure projecting from the virion envelope (3, 16, 19, 20, 25, 26, 35, 38). Second, HSV binds to CHO cells, apparently to glycosaminoglycan moieties, but this binding may not be sufficient to promote entry of HSV. The block to HSV replication in CHO cells has not been defined. Third, transformed cells expressing gD are resistant to HSV infection. Virus can adsorb to gD-expressing cells, but penetration is blocked (5; R. Johnson and P. G. Spear, submitted for publication). Although gD is dispensable for the initial adsorption of virus (25), gD probably interacts with a secondary cell surface receptor required for penetration. Interestingly, although CD41 is recognized as a receptor for the human immunodeficiency virus, sulfated polyanions block the binding of this virus to cells (29).

Several possibilities exist as to the significance of the interaction of HSV with cell surface heparan sulfate. The binding of virus to heparan sulfate may serve merely to concentrate the virus on the cell surface so as to facilitate binding to another receptor. Alternatively, interaction of a virion component with heparan sulfate may be necessary to enable some other virion-cell interaction to occur. For example, the neural cell adhesion molecule has both a heparin-binding domain and domains for interaction with other such molecules. Binding of heparan sulfate to the former domain facilitates homotypic interactions of the latter domains (9, 10).

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