Herpes Simplex Virus Infection and Propagation in a Mouse L Cell Mutant Lacking Heparan Sulfate Proteoglycans

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We have isolated a variant line of mouse L cells, termed gro2C, which is partially resistant to infection by herpes simplex virus type 1 (HSV-1). Characterization of the genetic defect in gro2C cells revealed that this cell line harbors a specific defect in the heparan sulfate synthesis pathway. Specifically, anion-exchange high-performance liquid chromatography of metabolically radiolabeled glycosaminoglycans indicated that chondroitin sulfate moieties were synthesized normally in the mutant cells, whereas heparin-like chains were absent. Because of these properties, we have used these cells to investigate the role of heparan sulfate proteoglycans in the HSV-1 life cycle. In this report, we demonstrate that the partial block to HSV-1 infection in gro2C cells occurs in the virus entry pathway. Virus adsorption assays using radiolabeled HSV-1 (KOS) revealed that the gro2C cell surface is a relatively poor target for HSV-1 in that virus attachment was 85% lower in the mutant cells than in the parental L cell controls. A portion of the 15% residual virus adsorption was functional, however, insofar as gro2C cells were susceptible to HSV-1 infection in plaque assays and in single-step growth experiments. Moreover, although the number of HSV-1 plaques that formed in gro2C monolayers was reduced by 85%, the plaque morphology was normal, and the virus released from the mutant cells was infectious. Taken together, these results provide strong genetic evidence that heparan sulfate proteoglycans enhance the efficiency of HSV attachment to the cell surface but are otherwise not essential at any stage of the lytic cycle in culture. Moreover, in the absence of heparan sulfate, other cell surface molecules appear to confer susceptibility to HSV, leading to a productive viral infection.

The genome of herpes simplex virus type 1 (HSV-1) encodes an elaborate array of at least 10 glycoproteins, 7 of which have been shown to decorate the virus envelope (reviewed in reference 21). As for all enveloped viruses, one or more of these proteins appear to facilitate the recognition and penetration of appropriate host cells. Some of the steps in the virus entry pathway have been dissected by the generation of HSV recombinants containing altered forms of one or more glycoproteins. These studies indicate that viral glycoproteins gB, gC, gD, and gH all contain determinants that influence virus adsorption or penetration (3, 6, 15). Although substantial progress is being made in identifying the viral determinants that influence virus entry, less is known about how these determinants recognize and interact with components of the host cell surface.

One area of significant progress in this regard has been the identification by Spear and coworkers of a role for heparan sulfate in the initial interaction of HSV-1 with the host cell (7, 20, 22, 26). Heparan sulfate is produced by virtually all animal cells and as such is a good candidate for the initial interaction of HSV with the cell surface. Evidence for the involvement of heparan sulfate in the initial events of herpesvirus infection has come from studies showing that soluble heparan sulfate, but not chondroitin sulfate, interferes with HSV infection in some cell types (17) and that enzymatic removal of heparan sulfate from the surface of host cells reduces infection (7, 26). Furthermore, radiolabeled herpesvirus attaches poorly to CHO cell mutants defective in glycosaminoglycan synthesis (20). In this study, the CHO mutant cell line pgsD-677, which is defective in heparan sulfate synthesis (14), was uninfected by HSV-1 (KOS) at multiplicities of infection (MOIs) as high as 150, suggesting a key role for heparan sulfate synthesis in the infection of CHO cells by HSV-1. Type-specific differences were also apparent in that HSV-2 (333) infected pgsD-677 cells relatively efficiently (10% of control). Mutant cells expressing undersulfated proteoglycans were also poor targets for virus infection, suggesting that the extent of sulfation may be a determinant in establishing the affinity of HSV-1 for the cell surface.

The role of proteoglycans in the herpesvirus life cycle has not been fully resolved by these studies, however, because CHO cells are one of the few cell types relatively nonpermissive to HSV infection. To overcome some of these limitations, we have isolated and characterized a murine L cell mutant, gro2C, that is defective in heparan sulfate proteoglycan synthesis. In this report, we demonstrate that gro2C cells are defective in HSV-1 adsorption, which results in a reduction in infection efficiency in comparison with parental L cell controls. However, gro2C cells can be infected by HSV-1 (KOS), and plaques that are indistinguishable from control cell plaques in both size and morphology form in gro2C monolayers. Furthermore, we show that virus assembly and egress are normal in gro2C cells, despite the absence of heparan sulfate biosynthesis. Taken together, these results show that heparan sulfate biosynthesis is not a requirement for a permissive HSV infection in mouse L cells. Moreover, in the absence of heparan sulfate, other cell surface molecules appear to confer susceptibility to HSV, leading to a productive viral infection.

MATERIALS AND METHODS

Materials. The parental cells used for all experiments were from the clone 1D line of LMtk− murine fibroblasts. Vero
cells used for determination of virus titers were obtained from S. McKnight. HSV-1 (KOS) was obtained from D. Coen (Harvard Medical School, Boston, Mass.). [35S]Sulfate (25 to 40 Ci/mmol) and d-[6-3H]glucosamine were obtained from ICN. [35S]Methionine was obtained from Dupont-New England Nuclear. Tissue culture reagents were obtained from Gibco Canada (Mississauga, Ontario, Canada).

Isolation of mutant cell lines. The procedure for isolating gro mutants was described previously (24). Briefly, murine L cells were grown in 100-mm-diameter plastic dishes containing Dulbecco modified Eagle medium (DME) and 10% fetal calf serum (FCS). Cells were infected with HSV-1 at an MOI of 1 to 3 PFU per cell. This level of infection resulted in the death of most cells within 96 h. About 1 in 106 of the original population of cells survived to form colonies.

Pulse-chase labeling experiments. Monolayers of L, gro2C, and gro29 cells were infected with vesicular stomatitis virus (VSV) Indiana strain at an MOI of 10. After 1 h, the virus was replaced and replaced with DME-10% FCS containing 100 μg of actinomycin D per ml to suppress host transcription during the course of the infection. At 4 h postinfection, cells were washed three times with methionine-free medium and labeled for 30 min with [35S]Methionine at 100 μCi/ml in methionine-free medium containing 5% dialyzed FBS and actinomycin D. At the completion of labeling, cells were either harvested immediately or washed three times and incubated in DME-10% FCS containing excess methionine for either 15 or 45 min. Cell extracts were prepared by washing the monolayer with cold phosphate-buffered saline (PBS) and then incubated for 15 min with cold lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate). Samples of each lysate were adjusted with concentrated sodium dodecyl sulfate (SDS) sample buffer to 0.0625 M Tris (pH 6.8)-2.3% SDS-5% β-mercaptoethanol-10% glycerol, heated at 100°C for 5 min, and subjected to electrophoresis in an SDS-10% polyacrylamide gel. Gels were fixed, dried, and exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) for autoradiography.

Isolation of radiolabeled virus. Monolayers of L cells were infected with HSV-1 (MOI = 10) and incubated for 2 h. At this time, the growth medium was replaced with DME containing [3H]thymidine (50 μCi/ml). After 2 days to allow virus egress, the extracellular medium was removed from infected-cell monolayers and subjected to low-speed centrifugation to pellet cell debris. The supernatant was sedimented at 10,000 × g for 2 h to pellet virions. This material was subjected to centrifugation and centrifuged through sucrose gradients formed in 50 mM NaCl-10 mM Tris (pH 7.8). Radiolabeled virus was removed from the gradient and diluted into PBS just prior to use. For determination of radioactivity in insoluble material, 10% of each fraction to be analyzed was added to 50 μg of bovine serum albumin followed by 1 ml of 10% cold trichloroacetic acid. Insoluble material was collected onto filters after 1 h, and radioactivity was measured by scintillation counting. For determination of virus titers, virus preparations were diluted serially with growth medium and used to inoculate monolayers of Vero cells growing in six-well dishes. Plaques were scored after 3 and 5 days.

Virus adsorption assays. The assays were modified from those described previously (26). Approximately 12 h prior to an experiment, 5 × 103 cells were plated into individual wells of a 96-well tissue culture dish. Radiolabeled virus was diluted in adsorption buffer (PBS containing 1% FCS, 0.1% glucose, and bovine serum albumin [fraction V; 5 mg/ml]). Cells were rinsed with cold adsorption buffer and inoculated with diluted radiolabeled virus for 3 h at 4°C. In some samples, heparan sulfate (50 μg/ml; Sigma) was added to the binding buffer to block viral adsorption. Following this incubation, the inoculum was removed and the monolayers were washed twice with cold adsorption buffer and once with cold PBS and then harvested with 100 μl of lysis buffer (PBS containing 1% SDS and 1% Triton X-100). The wells were rinsed with an additional 100 μl of lysis buffer, which was pooled with the original wash in scintillation vials for liquid scintillation spectroscopy.

Analysis of glycosaminoglycans. Biochemical labeling of glycosaminoglycans was performed by a modification of procedures described by Bame and Esko (1). Briefly, glycosaminoglycans were radiolabeled by incubating cells for 3 days with [35S]sulfate (10 μCi/ml) and d-[6-3H]glucosamine (20 μCi/ml) in DME-10% FCS modified to contain 10 mM sulfate and 1 mM glucose. The cells were washed three times with cold PBS and solubilized with 1 ml of 0.1 M NaOH at 25°C for 15 min. Samples were removed for protein determination. Extracts were adjusted to pH 5.5 by the addition of concentrated acetic acid and treated at 40°C for 12 h with 2 mg of pronase (Sigma) per ml in 0.32 M NaCl-40 mM sodium acetate (pH 5.5) containing shark cartilage chondroitin sulfate (2 mg/ml) as the carrier. For some experiments, portions of the radioactive material were treated for 12 h at 40°C with 10 μl of chondroitin ABC lyase, 0.5 U of heparitinase, or hyaluronidase. The radioactive products were quantified by chromatography on DEAE-Sepharose (Pharmacia LKB) by binding in 100 mM NaCl followed by elution with 0.7 M NaCl or precipitation with cetylpyridinium chloride where indicated (25).

For high-pressure liquid chromatography (HPLC) analysis, the glycosaminoglycan samples were desalted by precipitation with ethanol (1). Following centrifugation, the ethanol precipitates were suspended in 20 mM Tris (pH 7.4) and resolved by anion-exchange HPLC, using a TSK DEAE-3SW column (15 by 75 mm; Beckman Instruments). Proteoglycans were eluted from the column by using a linear 50 to 700 mM NaCl gradient formed in 10 mM KH2PO4 (pH 6.0). All buffers contained 0.2% Zwittergent 3-12 (Calbiochem) to extend the life of the column, as reported previously (1). The glycosaminoglycans in the peaks were identified by digestion of the sample with the relevant enzymes prior to chromatography.

Plaque assays. Mutant or control cells were inoculated with virus in DME-2% FCS for 1 h. After 6 to 48 h of incubation, samples of the cells and extracellular medium were harvested and the virus titers were determined. To do this, virus samples were diluted serially in DME-10% FCS and used to inoculate confluent monolayers of Vero cells growing in six-well dishes. After inoculation, the monolayers were rinsed and overlaid with either methylcellulose or growth medium supplemented with 0.1% pooled human immunoglobulin G. Plaques were scored after 3 and 5 days.

Comparison of the infection efficiencies of mutant and wild-type cells. Cells growing in monolayer were inoculated with HSV-1 (MOI = 3) for 1 h and incubated for 2 h. Cells were then removed from the dish with either trypsin or EDTA and replated in triplicate at approximately 100 colonies per dish in 100-mm-diameter dishes. Mock-infected cells were plated in a similar manner. After 7 to 10 days, colonies of cells growing on dishes were counted. The results reported are averages of two separate experiments counted in triplicate. Other experiments performed with
different virus stocks at MOIs ranging from 1 to 10 yielded similar results. Virus produced by infected cells in the assay dish was not in high enough concentration to kill adjacent colonies, as confirmed by experiments in which pooled human immunoglobulin G was included in the medium to neutralize free virus as soon as it was produced.

RESULTS

Isolation of heparan sulfate synthesis mutants. As part of a broad study to identify host cell molecules that facilitate HSV propagation, a genetic selection was used to isolate murine L cell lines capable of surviving exposure to HSV-1. To do this, cell monolayers were infected with HSV-1 and then incubated for 5 days to allow the monolayers to disintegrate. After 8 to 10 days, colonies of cells arose at the rate of approximately 1 in 10⁶. Stable clonal cell lines were established from independent colonies in three separate selections and have been propagated for more than a year. Cell variants were identified on the basis of a distinctive herpesvirus resistance phenotype; that is, monolayers of mutant cells were destroyed more slowly than control cells after exposure to HSV-1 at a low MOI. One cell line isolated in this manner, gro2C, was characterized to identify the underlying basis of the herpesvirus resistance phenotype.

Survival of gro2C cells after exposure to HSV-1. The relatively slow destruction of the gro2C cell monolayer after a low-multiplicity infection implied that gro2C cells were at least partially permissive for virus propagation and that HSV-1 was capable of spreading from cell to cell after infection. To investigate the possibility that gro2C cells were not infected efficiently, mutant and control L cells were incubated with HSV-1 at an MOI of 3 (relative to control L cells) for 2 h and replated at low density to allow for colony formation. Colonies formed after several days, indicating that some of the gro2C cells were capable of surviving exposure to HSV-1 during the initial inoculation. In these assays, 83% ± 4% of the control L cells died, whereas only 14% ± 5% of gro2C cells died (n = 5), which suggested that the majority of the gro2C cells escaped infection during the initial exposure to HSV. Alternatively, it may be the case that the infection aborted in some but not all cells, thereby allowing for cell survival.

Binding of radiolabeled HSV-1 to cells. To investigate the possibility that gro2C cells were not infected efficiently after exposure to HSV-1, monolayers of control L cells and gro2C cells were incubated with increasing concentrations of purified [3H]thymidine-labeled HSV-1 for 3 h at 4°C. Quantitative analysis of virus attachment (Fig. 1) showed that L cell monolayers adsorbed 1.5 x 10⁶ cpm at the highest concentration of input virus tested, whereas gro2C cells adsorbed only 1.5 x 10⁴ cpm. The addition of 50 µg of heparin per ml, which has been shown previously to interfere with both herpesvirus attachment and infection of permissive cells (7, 26), reduced cell-associated radioactivity by 90% in control L cells. By contrast, this treatment did not reduce the association of radioactivity with gro2C. Because it was clear that gro2C could be infected with HSV-1, the residual radioactivity associated with gro2C most likely represented a combination of radioactive material bound nonspecifically to the cell surface and virus bound in a manner that was not competed efficiently by the forms of commercial heparan sulfate used in these assays. Taken together, these results indicate that the gro2C cell surface is altered relative to the parental L cells such that gro2C cells no longer engage in an efficient interaction with HSV-1 particles.

Characterization of glycosaminoglycan synthesis in gro2C cells. It has been shown that HSV-1 (KOS) to parental L and gro2C cells. Virions labeled with [3H]thymidine were purified by centrifugation through sucrose gradients. Cells plated in 96-well dishes were exposed to virus samples for 3 h at 4°C in the presence or absence of heparan sulfate (50 µg/ml). Unbound material was removed by washing, cells were solubilized, and radioactivity was determined by scintillation counting. Protein concentration and cell number were determined at the beginning and end of the experiments, using additional control wells. The data shown, corrected for cell number, are the means of three determinations which did not vary by more than 10%. Experiments using three different virus preparations yielded similar results for the relative percent binding to gro2C versus control L cells. Symbols: ○, L cell controls; ■, gro2C cells; ●, L cells incubated with heparan sulfate; •, gro2C cells incubated with heparan sulfate.

FIG. 1. Adsorption of HSV-1 (KOS) to parental L and gro2C cells. Virions labeled with [3H]thymidine were purified by centrifugation through sucrose gradients. Cells plated in 96-well dishes were exposed to virus samples for 3 h at 4°C in the presence or absence of heparan sulfate (50 µg/ml). Unbound material was removed by washing, cells were solubilized, and radioactivity was determined by scintillation counting. Protein concentration and cell number were determined at the beginning and end of the experiments, using additional control wells. The data shown, corrected for cell number, are the means of three determinations which did not vary by more than 10%. Experiments using three different virus preparations yielded similar results for the relative percent binding to gro2C versus control L cells. Symbols: ○, L cell controls; ■, gro2C cells; ●, L cells incubated with heparan sulfate; •, gro2C cells incubated with heparan sulfate.
experiments, the glycosaminoglycan samples from control and gro2C cells were treated with chondroitin ABC lyase prior to chromatography. In these assays, all of the sulfated material from gro2C cell extracts was depolymerized, whereas in wild-type cells, only the material eluting at 0.6 M salt (chondroitin sulfate) was depolymerized (data not shown). These results suggest that all of the sulfated material in gro2C cells was chondroitin sulfate.

One possibility to account for the failure to detect sulfated heparin-like chains in gro2C cells could be that gro2C cells are incapable of synthesizing the alternating disaccharide characteristic of this glycosaminoglycan. To test this possibility, cell monolayers were incubated with [3H]glucosamine and [35S]sulfate to label both the carbohydrate chains and the sulfate moieties. After 3 days of labeling, glycosaminoglycan fractions from cell extracts and extracellular medium were pooled and analyzed by anion-exchange HPLC. Fractions eluting from the HPLC column were collected and counted by liquid scintillation spectroscopy. It can be seen in Fig. 3A that the control L cell glycosaminoglycans resolved into two major sulfated peaks containing predominantly heparan sulfate and chondroitin sulfate. The nonsulfated material eluting at 0.46 M salt was identified as hyaluronic acid by digestion with hyaluronidase prior to chromatography (data not shown).

By contrast, when gro2C cell glycosaminoglycans were subjected to HPLC analysis, there was essentially no heparan sulfate detected, despite the presence of normal or slightly elevated amounts of chondroitin sulfate (Fig. 3B). This can be seen as a complete absence of the carbohydrate and sulfate peaks eluting as heparan sulfate. An additional nonsulfated peak eluting at 0.2 M salt was not characterized further but may represent protein carbohydrate linkage regions accumulating in the absence of heparan sulfate.

### Table 1. Enzymatic digestion of glycosaminoglycans from cell extracts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Controla</th>
<th>+ Heparitinase</th>
<th>+ Chondroitin ABC lyase</th>
<th>Chondroitinase-resistant cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>2,039 ± 140</td>
<td>763 ± 58</td>
<td>1,050 ± 105</td>
<td>57.9</td>
</tr>
<tr>
<td>gro2C</td>
<td>817 ± 50</td>
<td>749 ± 33</td>
<td>14 ± 10</td>
<td>0.7</td>
</tr>
</tbody>
</table>

* Mutant gro2C and control L cells were labeled with [35S]SO₄ (50 μCi/ml) for 4 h in sulfate-free medium at 37°C. The cells were harvested, digested with pronase (2 mg/ml), and treated for 12 h at 45°C with 10 mU of chondroitin ABC lyase (Sigma) or 0.5 U of heparitinase as described in Materials and Methods. Radioactive products were quantified by cetylpyridinium chloride precipitation. The averages of two determinations in two experiments are shown.

* Averages of four samples, two from each enzyme digestion.

* Calculated relative to the total incorporation in the L cell controls.

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**FIG. 2.** Anion-exchange HPLC of cell-associated glycosaminoglycans derived from wild-type and gro2C cells. Cells were labeled with [35S]sulfate (50 μCi/ml) for 3 days. The medium was discarded, and the monolayers were washed extensively to remove any traces of medium from the cells. Radiolabeled glycosaminoglycans were released from cell-associated proteoglycans and collected by preparative anion-exchange chromatography and ethanol precipitation. A portion of [35S]-labeled glycosaminoglycans was chromatographed by anion-exchange HPLC, and the amount of radioactivity in each fraction was determined by liquid scintillation spectroscopy. The sulfated glycosaminoglycans in control L cells (○), gro2C cells (●) are shown. The peaks enriched for heparan sulfate (HS) and chondroitin sulfate (CS) are identified above the tracing. Hyaluronic acid is not sulfated and is therefore not detected by this procedure.

**FIG. 3.** Anion exchange HPLC of glycosaminoglycans derived from wild-type and gro2C cells and medium. Cells were labeled with 10 μCi each of [35S]sulfate (●) and [3H]glucosamine (○) per ml for 3 days. Radiolabeled glycosaminoglycans were released from cell and medium proteoglycans and collected by preparative anion-exchange chromatography and ethanol precipitation. A portion of [35S]-labeled glycosaminoglycans was chromatographed by anion-exchange HPLC, and the amount of radioactivity in each fraction was determined by liquid scintillation spectroscopy. The dotted line in panel A represents the salt gradient used for elution. (A) Control L cells; (B) gro2C cells. The peaks enriched for hyaluronic acid (HA), heparan sulfate (HS), and chondroitin sulfate (CS) are identified above the tracing. Note the change in scale for panels A and B.
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FIG. 4. VSV G protein synthesis and processing in mutant and control L cells. Cell monolayers were infected with VSV and incubated for 4 h in the presence of actinomycin D to suppress cellular transcription. To label viral proteins, [35S]methionine (200 µCi/ml) was added to the cells for 15 min. Monolayers were then rinsed and either harvested immediately (A) or incubated for an additional 15 (B) or 45 (C) min of chase. Cell extracts were prepared by incubating the monolayers with cold lysis buffer, and the nuclei were removed by centrifugation. Samples of extract from control L cells, gro2C mutants, and gro29 cells were resolved by SDS-polyacrylamide gel electrophoresis and visualized by fluorography. The positions of the VSV proteins are identified at the left.

Glycoprotein processing and transport in gro2C cells. It is well established that defects in glycoprotein processing or secretion can interfere with HSV propagation, as has been demonstrated for a variety of somatic cell mutants (2, 4, 24). Moreover, a previously isolated BHK cell mutant, RicR14, which is partially herpesvirus resistant, has been shown to express undersulfated heparan sulfate proteoglycans on the cell surface as well as defects in N glycosylation (5). To test whether the gro2C mutant contained a second defect in protein glycosylation or secretion that could account for its apparent herpesvirus resistance phenotype, we analyzed the synthesis and processing of the well-characterized glycoprotein VSV G. VSV G protein was chosen for these assays because its transport and glycoprotein processing have been characterized in a wide variety of previously existing somatic cell mutants (23). Monolayers of L cell controls, gro2C, and the secretion mutant gro29 (2, 24) were exposed to VSV and examined at 4 h postinfection by a pulse-chase labeling analysis. Infected cells were incubated for 15 min with [35S]methionine to label viral proteins. Proteins were then examined immediately postlabeling or incubated for an additional 15 or 45 min to allow the VSV G protein to traverse the secretory organelles prior to harvest (Fig. 4). It can be seen that all three cell lines were susceptible and permissive to VSV and synthesized normal amounts of glycosylated VSV G glycoprotein during the labeling period. After 15 min in L and gro2C cells, VSV G protein was transported from the endoplasmic reticulum to the Golgi complex. This transport was accompanied by oligosaccharide modifications, detected as changes in the relative mobility of VSV G protein in the gel (compare Fig. 4A and B). By contrast, there was no apparent flux of G protein in the gro29 secretory mutant, which serves as a control to denote the position of underprocessed G protein accumulating under conditions of reduced secretion (24). After 45 min of chase, VSV G proteins synthesized by L and gro2C cells were fully processed, whereas there was little evidence for processing in gro29 cells. We conclude from these results that gro2C is normal with regard to N glycosylation and as such is distinct from other well-characterized glycosylation mutants shown previously to interfere with HSV propagation.

Analysis of virus plaques in mutant cell monolayers. The isolation of permissive cells lacking heparan sulfate proteoglycan synthesis allowed us to test whether heparan sulfate proteoglycan synthesis is required for the production of HSV progeny. To determine whether HSV-1 plaques form on gro2C cell monolayers, mutant and control cells were exposed to HSV-1 at different concentrations and incubated for several days to allow for plaque formation (Fig. 5). In these assays, the number of plaques on gro2C monolayers was reduced by 85% ± 5% (n = 3) compared with parental L cells, consistent with the partial herpesvirus resistance phenotype first observed for gro2C cell monolayers. Interestingly, the gro2C plaques were similar in size and morphology
to L cell plaques, indicating that there was no impediment to infectious center formation or to virus propagation and spread from cell to cell. It is also noteworthy that the number of gro2C plaques increased with virus concentration such that the entire monolayer was destroyed at high virus concentrations. This result indicates that every cell in the gro2C monolayer was susceptible to infection. It is likely, therefore, that the reduction in plaque number on gro2C monolayers at each concentration of input virus is caused by a failure to efficiently infect the gro2C cells during the initial exposure to the virus.

The data so far indicated that HSV-1 was able to spread from cell to cell in gro2C cell monolayers. To test whether HSV-infected gro2C cells were able to release infectious virions, growth curves of intracellular and extracellular HSV-1 were generated (Fig. 6). When L cells were infected with HSV-1 at an MOI of 10 (Fig. 6A), intracellular infectious virus was detected by 5 to 7 h postinfection, and infectious virus was released from the cell by 7 to 9 h postinfection. A similar pattern of accumulation of intracellular virus and extracellular virus was observed for gro2C cells when they were infected with the same amount of virus (Fig. 6B). Taken together, these results show that the HSV-1 life cycle is not impeded by the failure of the gro2C host cells to synthesize heparin-like molecules.

To test whether the progeny virus produced by mutant gro2C cells was similar to wild-type virus, monolayers of control L cells and gro2C cells were infected with progeny virus released from gro2C cells. In these assays, there was an 85% reduction in plaque formation on gro2C cells compared with control L cell monolayers (data not shown). Thus, it appeared that the virus produced by gro2C cells was similar to wild-type virus in its ability to distinguish between control and gro2C cell monolayers in the plaque assay.

**DISCUSSION**

This report describes the isolation and characterization of a mammalian cell mutant that is defective in heparan sulfate biosynthesis. Although it cannot be concluded with certainty that gro2C synthesizes no heparan sulfate chains, the assays used for this study were sensitive enough to detect 0.05% or less of the normal complement of heparan sulfate. It has been estimated that typical concentrations of heparan sulfate proteoglycans on the cell surface of a variety of cell types are in the range of $10^5$ to $10^6$ molecules per cell (8) and that $10^3$ heparan sulfate proteoglycans present on the surface of a cell would cover the cell surface if the heparan sulfate chains were extended and mobile around the anchored core proteins (27). These estimates indicate that the cell surface of gro2C must be largely devoid of heparan sulfate.

Despite this deficiency, the cells are reasonably good targets for infection, considering that a >99.95% reduction in heparan sulfate expression reduces the infection efficiency of HSV-1 by only 85% (Fig. 5). These results suggest that HSV-1 need not interact specifically with heparan sulfate to gain entry into mouse fibroblasts. In this regard, it has been shown that the most likely candidate for the initial interaction with heparan sulfate is the viral glycoprotein gC, which is not essential for infectivity in culture. However, its presence in the virion enhances infectivity by a factor of 10 (7). It is noteworthy that we also observed an 8- to 10-fold reduction in infectivity in the absence of heparan sulfate (Fig. 5). Taken together, these results suggest that the principal role of gC during infection in culture is to interact with heparan sulfate to promote efficient virus entry.

Although the events of the virus entry pathway have not yet been well characterized, it is clear that at least three HSV glycoproteins, gB, gD, and gH, are essential for virus infection (3, 15, 16, 19). It may be the case that one or more of these viral glycoproteins engages a second cell surface receptor to facilitate virus entry. It has been proposed that gD may bind a cell surface component (10–12), which could serve to anchor virions at the surface or promote subsequent events such as fusion with the plasma membrane. Our results showing that gro2C cells can be infected by HSV-1 are consistent with a mechanism that allows the virus to interact productively with a second receptor without first interacting with heparan sulfate. This second interaction is apparently strong enough to account for the residual 15% infectivity demonstrated for gro2C cells in the absence of heparan sulfate.

It is clear from our studies and those of others (20) that soluble heparan sulfate is incapable of linking the virus to the host cell so as to overcome the lack of endogenously synthesized heparan sulfate (Fig. 1). This finding is consistent with previous results which showed that [35S]sulfate-labeled proteoglycans isolated from the medium of human fibroblast cultures are not incorporated into the extracellular matrix when added to unlabeled cell cultures (9). These results indicate that the matrix proteoglycans are not derived from the proteoglycans present in the medium and that an interaction between polysaccharide chains and matrix com-
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ponents is not sufficient for incorporation of proteoglycans into the matrix. This view is consistent with a model of virus entry wherein HSV virions attach to heparan sulfate or a molecule associated with heparan sulfate that arrives at the cell surface via endogenous synthesis. This model also implies that the functional heparan sulfate moieties will be found covalently linked to proteins embedded in or in close contact with the plasma membrane.

What is the likelihood that another cell surface molecule such as chondroitin sulfate could substitute for heparan sulfate to promote virus entry into gro2C cells? Heparan sulfate is composed of a repeating disaccharide of N-acetyl- or N-sulfoglucosamine and a hexuronic acid, whereas chondroitin sulfate consists of N-acetylgalactosamine and hexuronic acid. The polysaccharide of heparin is extensively modified through sulfation of glucosamine units and by N-deacetylation and N sulfation, leading to the formation of distinctive structural features. The structural complexity of heparin and heparan sulfate derives from the fact that the modification reactions tend to be incomplete (18), and it is much more extensive than for chondroitin sulfate. If HSV virions rely on a particular structural feature of heparan sulfate to stabilize them at the cell surface, it seems unlikely that chondroitin sulfate could serve this role in the absence of heparan sulfate. Support for this hypothesis comes from studies which show that CHO cells deficient in both chondroitin sulfate and heparan sulfate bind only slightly less virus than do cells with defects in heparan sulfate alone (20). Moreover, HSV-1 infection is not reduced in the presence of high concentrations of chondroitin sulfate chains (17), and the treatment of the cell surface with chondroitin lyase does not significantly reduce HSV-1 plaque formation on HEp-2 cells (26). Unfortunately, a direct assessment of the ability of chondroitin sulfate to serve as a receptor has not yet been possible because no cell lines with specific defects in chondroitin sulfate synthesis have been isolated.

In several respects, the gro2C phenotype resembles that of the CHO mutant cell line pgsD-677 (14). Both cell lines are defective in heparan sulfate synthesis and show some similarities with regard to their interactions with HSV. Whereas both cell lines are infected by HSV-2 (20, 23a), only gro2C cells can be infected by HSV-1. We are intrigued by the differences observed for gro2C versus pgsD-677 cells and propose that the removal of heparan sulfate from these cells has uncovered cell type differences in the cell surface molecules that facilitate the entry of HSV-1. Mouse L cells appear to express receptors for both HSV-1 and HSV-2 for which prior binding to heparan sulfate is not essential. By contrast, HSV-1 appears to gain entry to CHO cells via a molecule that functions poorly for virus entry without the help of the heparan sulfate interactions. It is intriguing to speculate that the relatively nonpermissive CHO cells have lost a functional receptor for HSV-1. If the entry of HSV-1 into CHO cells is via an alternative pathway, it may be that this pathway renders the virus unable to initiate infection efficiently. We would also infer from these results that the cellular components used by HSV-2 to gain entry to CHO and L cells are functional in the absence of heparan sulfate.

Characterization of the role of heparan sulfate proteoglycans in the HSV life cycle was also extended to postinfection events. Although virus attachment assays revealed that gro2C cells are relatively inefficient targets for stable virus attachment, one-step growth experiments show that virus gene expression and subsequent virus assembly and egress occur normally after virus uptake (Fig. 6). Moreover, plaque assays reveal that all of the gro2C cells are ultimately susceptible to infection: increasing the input plaque increases the number of plaques formed. Taken together, these results provide strong evidence that once a gro2C cell is infected, virus propagation ensues unperturbed by the virtual absence of heparan sulfate moieties in the host cell. This finding implies that none of the 10 herpesvirus glycoproteins identified need to be modified by the addition of heparan sulfate chains during virus propagation. Moreover, virions do not require heparan sulfate in the cell or medium to become infectious.

The most striking observation to come from this study is that gro2C cells, like pgsD-677 cells, have a specific defect in heparan sulfate polymerization and are poor targets for the stable attachment of HSV. These results provide strong evidence that heparan sulfate proteoglycans play a major role in facilitating herpesvirus infection in cells normally expressing these molecules. This conclusion is strengthened by the fact that the two mutant cell lines arose by different selection procedures: pgsD-677 were screened for a reduction in sulfate incorporation (14), whereas gro2C were selected for the ability to survive exposure to HSV-1. This fact virtually eliminates the possibility that a second mutation that could account for the herpesvirus resistance phenotype arose fortuitously in both cell lines during selection. In this regard, we have found that a high percentage of the cell lines isolated by the herpesvirus selection procedure are proteoglycan synthesis mutants. Because of the permissivity of mouse L cells to a variety of herpesviruses, these cellular mutants should be useful tools for identifying and characterizing viral glycoprotein-proteoglycan interactions. Moreover, the availability of glycoprotein-deficient herpesviruses should facilitate these investigations.

The results presented in this study suggest that HSV can be a powerful selective agent for identifying cellular components that are critical to the virus entry pathway. Furthermore, we are intrigued that the cell lines isolated in this way are killed when they are rechallenged with virus, which suggests that a second round of selection could be used to isolate mutants that are no longer susceptible to infection. Ideally, these cells would be defective in expressing additional components of the cellular machinery used by the virus to gain entry to the cell and could be useful for identifying these molecules.

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