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Sequential Isolation of Proteoglycan Synthesis Mutants by Using Herpes Simplex Virus as a Selective Agent: Evidence for a Proteoglycan-Independent Virus Entry Pathway

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A novel mouse L-cell mutant cell line defective in the biosynthesis of glycosaminoglycans was isolated by selection for cells resistant to herpes simplex virus (HSV) infection. These cells, termed sog9, were derived from mutant parental gro2C cells, which are themselves defective in heparan sulfate biosynthesis and 90% resistant to HSV type 1 (HSV-1) infection compared with control L cells (S. Gruenheid, L. Gatzke, H. Meadows, and F. Tufaro, J. Virol. 67:93–100, 1993). In this report, we show that sog9 cells exhibit a 3-order-of-magnitude reduction in susceptibility to HSV-1 compared with control L cells. In steady-state labeling experiments, sog9 cells accumulated almost no [^{35}S]sulfate-labeled or [6-^{3}H]glucosamine-labeled glycosaminoglycans, suggesting that the initiation of glycosaminoglycan assembly was specifically reduced in these cells. Despite these defects, sog9 cells were fully susceptible to vesicular stomatitis virus (VSV) and permissive for both VSV and HSV replication, assembly, and egress. HSV plaques formed in the sog9 monolayers in proportion to the amount of input virus, suggesting the block to infection was in the virus entry pathway. More importantly, HSV-1 infection of sog9 cells was not significantly reduced by soluble heparan sulfate, indicating that infection was glycosaminoglycan independent. Infection was inhibited by soluble gD-1, however, which suggests that glycoprotein gD plays a role in the infection of this cell line. The block to sog9 cell infection by HSV-1 could be eliminated by adding soluble dextran sulfate to the inoculum, which may act by stabilizing the virus at the sog9 cell surface. Thus, sog9 cells provide direct genetic evidence for a proteoglycan-independent entry pathway for HSV-1, and results with these cells suggest that HSV-1 is a useful reagent for the direct selection of novel animal cell mutants defective in the synthesis of cell surface proteoglycans.

Herpes simplex virus (HSV) is an important human pathogen. Its large DNA genome comprises genes encoding a variety of structural proteins, including at least 11 glycoproteins (1, 2, 6, 8, 30, 31, 40, 49, 56, 57). Many of the glycoproteins have been shown to decorate the virion envelope, and at least four of them, gB, gD, gH, and gL (9, 10, 14, 15, 19, 20, 22, 23, 27, 29, 30, 34, 39, 44, 50, 51), play an essential role in the virus entry pathway in cultured cells. While many laboratories have studied roles of glycoproteins in the viral life cycle, relatively little is known about the manner in which the glycoproteins facilitate the processes of virus entry, assembly, and egress.

The current model for HSV infection predicts that a spike protein, probably gC or gB, embedded in the envelope of the virion contacts glycosaminoglycan moieties on cell surface proteoglycans, which are ubiquitous components of mammalian cells and tissues (10, 12, 24–26, 37, 54, 59, 60, 63). Evidence for this interaction stems from observations that soluble heparin or heparan sulfate and, in some instances, chondroitin sulfate (5, 24) can reduce HSV infection by over 90% if these molecules are present during inoculation. Because soluble glycosaminoglycans do not appear to reduce the infectivity of the virion itself, it is likely that the soluble glycosaminoglycans act as competitive inhibitors of virus attachment. Artificial reduction of the cell surface concentration of heparan sulfate by enzymatic digestion also reduces HSV infection, which suggests that heparan sulfate, or a molecule with which heparan sulfate associates, plays a role in HSV infection (63). Furthermore, animal cell mutants with defects in GAG synthesis show either complete (54) or partial (24) resistance to HSV infection, and cells selected for HSV resistance have been shown in some instances to have a specific defect in heparan sulfate synthesis (24). The situation is complicated, however, by reports that the major HSV spike glycoprotein, gC, which is capable of binding to purified heparin, is not essential for infection (26, 37, 52). Although a reduction in the specific infectivity of gC-deficient virus has been ascribed to the loss of heparin-binding ability, there has been no direct demonstration that this is the case. The possibility that another virion glycoprotein may provide a redundant function to gC is supported by the observation that infection by gC-deficient HSV type 1 (HSV-1) virions can be inhibited effectively by soluble heparin (26). Moreover, a report that virions devoid of both gB and gC do not attach to cells as efficiently as do virions deficient in gC alone suggests that glycoprotein gB, which also binds to purified heparin, may function in the initial stages of attachment to cells (25).

Many reports on HSV entry have provided evidence for a second phase of attachment to host cells in culture. In most instances, soluble heparan sulfate or chondroitin sulfate does not inhibit infection completely, suggesting that there is a second receptor to which the virion can attach. Direct evidence for a second receptor was provided by Sears et al. (52), who showed that MDCK cells contain a gC-dependent and a gC-independent receptor, and more recently by Subramanian and colleagues (58), who reported that swine testis cells have low

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susceptibility to HSV infection despite having an apparently normal complement of cell surface glycosaminoglycans. Soluble forms of the essential glycoprotein gD have also been shown to interfere with infection and appear to interact with a saturable molecule on the host cells (33). In this regard, the mannose-6-phosphate receptor (M6PR) has been proposed as a candidate for this receptor on the basis of evidence that glycoprotein gD is modified by mannose-6-phosphate moieties on a fraction of its oligosaccharides, thereby providing a ligand for attachment (7). Antibodies to M6PR reduce infection, and ligands capable of interacting with M6PR inhibit infection presumably by competing for receptor sites on the cell surface. On the other hand, M6PR is apparently not essential for infection of susceptible mouse cells (32). Thus, the role, if any, of the M6PR in HSV-1 infection will need to be investigated further.

As part of a broad study to identify and characterize host cell factors which facilitate HSV infection, mouse L-cell mutants were isolated by selecting for HSV-1-resistant cells (24). One of the useful features of mouse L cells is that plaques form in the monolayer in direct proportion to the amount of input virus. This property allows for a sensitive means of assessing the efficiency of infection under a variety of experimental conditions. We have shown previously that HSV infection of the mutant cell line termed gro2C, which synthesizes chondroitin sulfate but not heparan sulfate, was 90% resistant to infection relative to parental control cells and was inhibited when soluble heparan sulfate or chondroitin sulfate was present during virus adsorption. This suggested that infection of gro2C cells was mediated by glycosaminoglycans other than heparan sulfate. We reasoned that it should be possible to select for a variant of gro2C cells which is less susceptible to infection and which would be defective in additional components of the virus entry pathway. Here, we report the isolation of a novel HSV-1-resistant cell line isolated by HSV selection which shows a 3-order-of-magnitude decrease in susceptibility to HSV-1 infection compared with control L cells. This cell line, termed sog9, harbors additional defects in the glycosaminoglycan synthesis pathway such that they fail to synthesize any glycosaminoglycans. We present evidence that HSV-1 infection of sog9 cells is facilitated by novel virus-host interactions that are glycosaminoglycan independent.

MATERIALS AND METHODS

Materials. The parental L cell used for all experiments was the clone 1D line of LMtk$^-$ murine fibroblasts. The HSV-1 strain KOS was obtained from D. Coen (Harvard Medical School, Boston, Mass.). HSV-1 gC$^-$ virus, HSV-1(KOS) ΔgC2-3, was obtained from C. Brandt (University of Wisconsin, Madison). The HSV-2 strain G was obtained from S. Sacks (University of British Columbia, Vancouver, Canada). The HSV-1 strains F and MP were obtained from B. Roizman (University of Chicago, Chicago, Ill.). Monoclonal antibodies specific for HSV-1 ICP4 and HSV-1 gB were obtained from M. Zwieg (National Cancer Institute, Frederick, Md.). [^{35}S]sulfate, D-[6-^{3}H]glucosamine, and [^{35}S]methionine were obtained from ICN (St. Laurent, Quebec, Canada). Heparan sulfate (from bovine intestinal mucosa) was obtained from Sigma (St. Louis, Mo.). Dextran sulfate (average molecular weight, 500,000) was from Pharmacia (Piscataway, N.J.). All tissue culture reagents (Gibco) and dishes (Nunc) were from Canadian Life Technologies (Burlington, Ontario, Canada).

Isolation of mutant cell lines. The procedure for isolating gro mutants was described previously (61). Briefly, murine L cells were grown in plastic dishes (100-mm diameter) containing Dulbecco modified Eagle medium and 10% fetal calf serum (DMEM-FCS). Cells were infected with HSV-1 at a multiplicity of infection (MOI) of 1 to 3 PFU per cell. This resulted in the death of most cells within 96 h. About 1 in 10^6 of the original population of cells survived to form colonies. In order to isolate clonal cell lines, colonies were harvested and cells were plated at a cell density of 0.3 cell per well in 96-well dishes. Wells containing single cells were identified, and the cells were grown up as clonal cell lines and tested for their capacity to support viral infection.

Plaque assays. Mutant or control cell monolayers growing in six-well-cluster dishes were inoculated with serial 10-fold dilutions of virus. After a 1-h adsorption period at 37°C, the inoculum was removed and the cells were thoroughly washed three times with phosphate-buffered saline (PBS) to remove unbound virus. The cells were then overlaid with DMEM containing 4% FCS and 0.1% pooled human immunoglobulin G (IgG) (HSV-1 and HSV-2) or 0.8% agarose (vesicular stomatitis virus [VSV]). Plaques were counted and visualized after 24 (VSV), 72 (HSV-2), or 96 [HSV-1 and HSV-1(KOS)ΔgC2-3] h postinfection by fixing and staining the cells for 5 min with 5% methylene blue in 70% methanol.

Cell labeling experiments. Monolayers of mutant and control L cells growing in 60-mm culture dishes were inoculated with serial 10-fold dilutions of HSV-1. After a 1-h adsorption period at 37°C, the inoculum was removed and the monolayers were rinsed with warm PBS; afterwards, fresh medium was added to the dishes. At 5 h postinfection, cells were washed three times with methionine-free medium and labeled for 60 min with 50 μCi of [^{35}S]methionine per ml in methionine-free medium containing 5% dialyzed FCS. At the completion of labeling, cells were harvested by washing the monolayer with cold PBS and incubated for 15 min with 1 ml of cold lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% Na-deoxycholate). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer was added to portions of lysates, and these were subjected to SDS-PAGE as described elsewhere (36). Following electrophoresis, gels were fixed and dried, and autoradiography was performed.

Indirect immunofluorescence. Cells were grown on glass coverslips for 3 days and infected with various concentrations of HSV-1. At 5 h postinfection, cells were rinsed with PBS and fixed with 2% formaldehyde in PBS for 10 min at 20°C. Cells were permeabilized in PBS–1% bovine serum albumin (BSA)–0.2% Triton X-100 for 3 min. Permeabilization was stopped by rinsing the cells in PBS–1% BSA. The cells were then incubated with anti-ICP4 monoclonal antibody diluted in PBS–1% BSA at 20°C for 1 h. Monolayers were washed extensively and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG diluted in PBS–1% BSA for 30 min. Coverslips were then washed and mounted in 50% glycerol–100 mM Tris, pH 7.8. Images were photographed with a Zeiss Axiophot microscope with epifluorescence optics.

Preparation of radiolabeled HSV-1. Monolayers (90% confluent) of Vero cells were inoculated with HSV-1 at an MOI of 0.1. After a 1-h adsorption period at 37°C, the inoculum was removed and the cells were washed with PBS and labeled for 48 h with 25 μCi of [^{35}S]methionine in methionine-free medium containing 5% dialyzed FCS and 10% DMEM (with methionine). The medium was collected and centrifuged at 600 × *g* for 10 min at 4°C to remove cells and cellular debris. Aliquots (10 ml) of medium were layered over a 3-ml cushion of 15% sucrose (wt/wt, in PBS) in an SW41 centrifuge tube. Tubes were centrifuged at 100,000 × *g* for 1 h in an SW41 rotor. The supernatant was discarded, and the pellets were suspended overnight in 1 ml of sterile DMEM–1% BSA–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.4). The typical specific activity of such preparations was 0.2 to 0.5 cpm/PFU.

Virus adsorption assays. Confluent monolayers of mutant and control L cells (containing approximately 5 × 10^5 cells) growing in 24-well dishes were rinsed with PBS and incubated for 1 h at 37°C in adsorption medium (DMEM–1% BSA–20 mM HEPES [pH 7.4]). Dishes were removed from the incubator and placed on ice. The medium was removed from the monolayers, and 100 μl of radiolabeled virus diluted in adsorption medium was added to the wells and incubated for 1 h on ice. In some experiments, the radiolabeled virus was diluted in adsorption medium containing various concentrations of dextran sulfate. Following this adsorption period, the monolayers were rinsed four times with 0.5 ml of PBS prior to solubilization in cold lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 1% Na-deoxycholate). Lysates were added to a scintillation vial, and the radioactivity associated with the monolayers was determined by liquid scintillation spectroscopy. Data presented represent the averages of at least four determinations.

Analysis of glycosaminoglycans. Biochemical labeling of glycosaminoglycans was performed by a modification of procedures described previously by Bame and Esko (3). Briefly, glycosaminoglycans were radiolabeled by incubation of cells for 3 days with 10 μCi of [^{35}S]sulfate per ml and 20 μCi of D-[6-^{3}H]glucosamine per ml in DMEM-FCS modified to contain 10 μM sulfate and 1 μM 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 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 carrier, at 40°C for 12 h. For some experiments, portions of the radioactive material were treated for 12 h at 40°C with 10 mU of chondroitinase ABC lyase or 0.5 U of heparitinase. The radioactive products were quantified by chromatography on DEAE-Sephacel (Pharmacia) by binding in 100 mM NaCl followed by elution with 0.7 M NaCl.

For high-pressure liquid chromatography (HPLC) analysis, the glycosaminoglycan samples were desalted by precipitation with ethanol (3). Following centrifugation, the ethanol precipitates were suspended in 20 mM Tris, pH 7.4, and resolved by anion exchange HPLC with a TSK DEAE-3SW column (15 by 75 mm; Beckman Instruments, Mississauga, Ontario, Canada). Proteoglycans were eluted from the column with a linear 50 to 700 mM NaCl gradient formed in 10 mM KH_2PO_4 (pH 6.0). All buffers contained 0.2% Zwittergent 3-12 (Calbiochem, La Jolla, Calif.) to extend the life of the column. The glycosaminoglycans in the peaks were identified by digestion of the sample with the relevant enzymes prior to chromatography.

TABLE 1. Relative infectivities of virus strains on control and mutant cell lines

Virus	Relative infectivity (%) on cells of indicated line[a]		
	L (control)	gro2C	sog9
HSV-1(KOS)	100	10	0.3
HSV-1(KOS)ΔgC2-3	100	10	0.8
HSV-1(F)	100	20	1
HSV-1(MP)	100	25	2
HSV-2(G)	100	3.3	1
VSV(Indiana)	100	78	175

[a] Titers of serial 10-fold dilutions of virus stocks grown in Vero cells were determined on cell monolayers. Results from at least three determinations were averaged and expressed as percentages of control L cell infection. Titers on sog9 cells varied by up to 25%.

Plaque inhibition assays. Monolayers (95% confluent) of L or gro2C cells plated in 6-well-cluster dishes were rinsed twice with PBS and inoculated with HSV-1 diluted in DMEM previously mixed with various concentrations of heparan sulfate or dextran sulfate. After a 60-min adsorption period at 37°C, the inoculum was removed and the cells were washed three times with PBS to remove unbound virus. The cells were then overlaid with DMEM containing 4% FCS and 0.1% pooled human IgG. Plaques were visualized and counted after 3 to 4 days by fixation and staining of the cells for 5 min with 5% methylene blue in 70% methanol.

Inhibition of plaque formation by soluble gD-1. A soluble truncated form of HSV-1 gD, gD-1(306t) (13), was diluted in DMEM–5% FCS to 0 to 500 μg/ml and incubated with monolayers of sog9 and control L cells for 1 h at 37°C. After this 1-h incubation, HSV-1 diluted in the appropriate concentration of gD-1(306t) was added to the monolayers and allowed to adsorb to the cells for 1 h at 37°C. The virus and gD-1(306t) were removed from the monolayers, and medium containing 0.1% pooled human IgG was added to allow for plaque formation.

RESULTS

Isolation and characterization of infection in HSV-resistant cell lines. The gro2C cell line was used as the parental cell for the isolation of new cell variants exhibiting increased resistance to HSV-1 infection. gro2C cells are 90% compared with control L cells resistant to HSV-1 infection and unable to synthesize heparan sulfate glycosaminoglycans (24). We reasoned that cells deficient in additional cell surface molecules to which HSV-1 can bind would be isolated by sequential selection for cells refractory to infection. This strategy was used to isolate a variant clonal cell line, termed sog9, which showed approximately 95% resistance to HSV-1 infection compared with parental gro2C cells (Table 1). They were also cross-resistant to HSV-2, but not to the unrelated enveloped virus VSV.

To investigate the underlying basis of HSV resistance, confluent monolayers of sog9 cells were incubated with increasing amounts of HSV-1 for 1 h, and the number of plaques on each monolayer was determined after 3 days (Fig. 1). In these assays, plaques formed in the sog9 monolayers in direct proportion to the amount of input virus, indicating that every cell in the monolayer was ultimately susceptible to infection and could lead to plaque formation. Moreover, infected sog9 cells yielded progeny virus that was indistinguishable from wild-type virus in terms of the ability to infect L, gro2C, and sog9 cells (data not shown); thus, sog9 cells were likely infected by wild-type virus in the inoculum. After infection, however, substan-

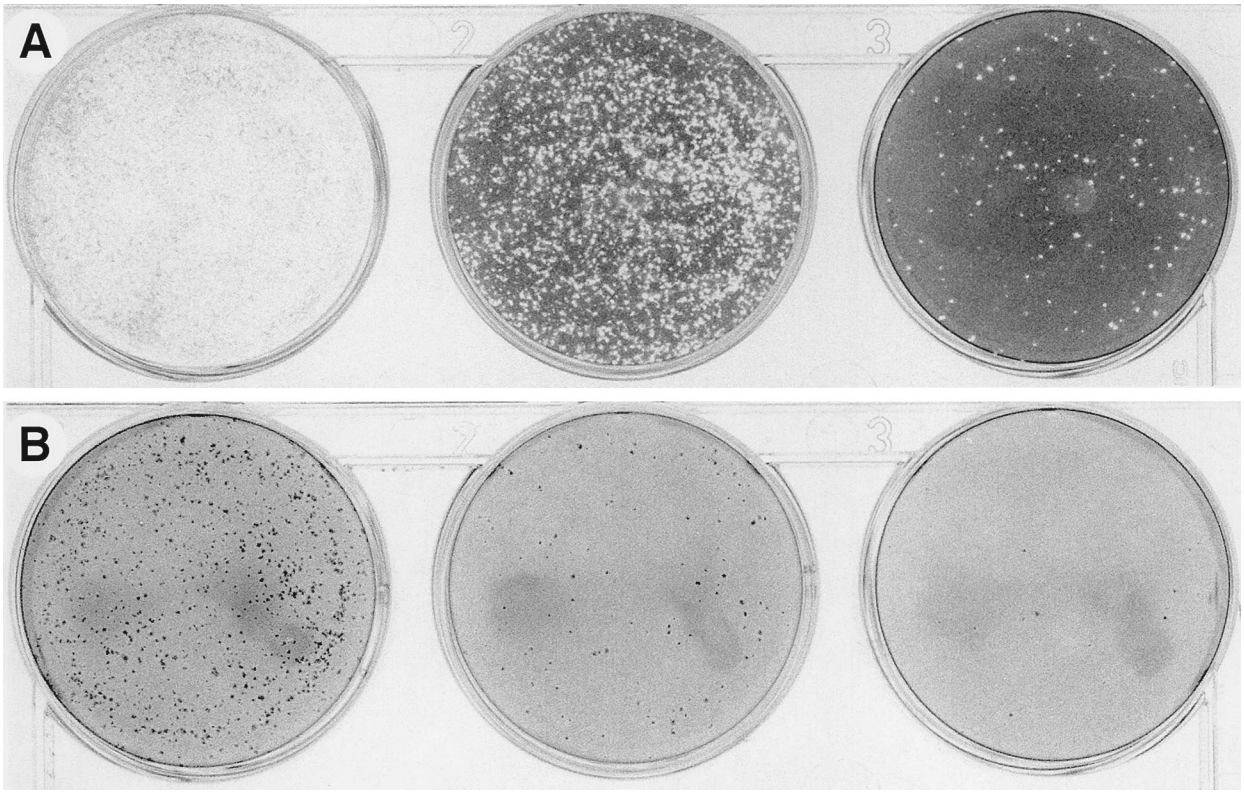

FIG. 1. Plaque-forming efficiency of HSV-1 on parental gro2C and sog9 cells. Serial 10-fold dilutions (left to right) of HSV-1(KOS) were plated on parental L-cell and gro2C mutant cell monolayers and allowed to adsorb for 1 h. Following incubation, the inoculum was replaced with pooled human IgG to allow plaques to form. At 4 days postinfection, the cells were rinsed and fixed with 5% methylene blue in 70% methanol. Plaques appear as clear areas on a dark background for gro2C cells (A) and as dark areas on a light background for sog9 cells (B).

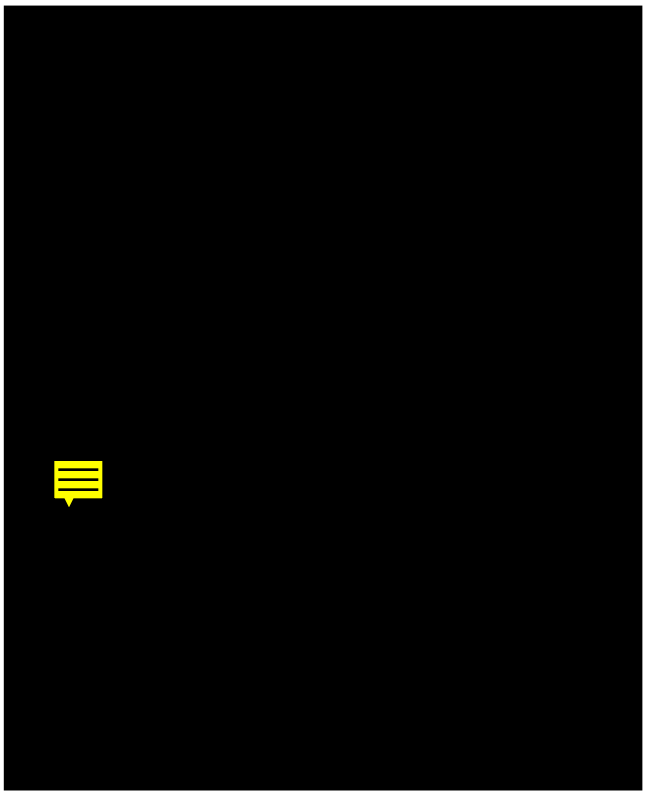

FIG. 2. HSV-1(KOS) plaque morphology on gro2C and sog9 monolayers. Monolayers of gro2C cells and sog9 cells were infected with HSV-1(KOS) at a low MOI. After 1 h of adsorption at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. After 4 days to allow for plaque formation, monolayers were washed with PBS and fixed for 5 min with 5% methylene blue in 70% methanol. The excess stain was washed away with distilled water, and plaques were photographed with a Leitz inverted microscope equipped with a Wild Leitz 35-mm camera. (A) gro2C cells; (B) sog9 cells.

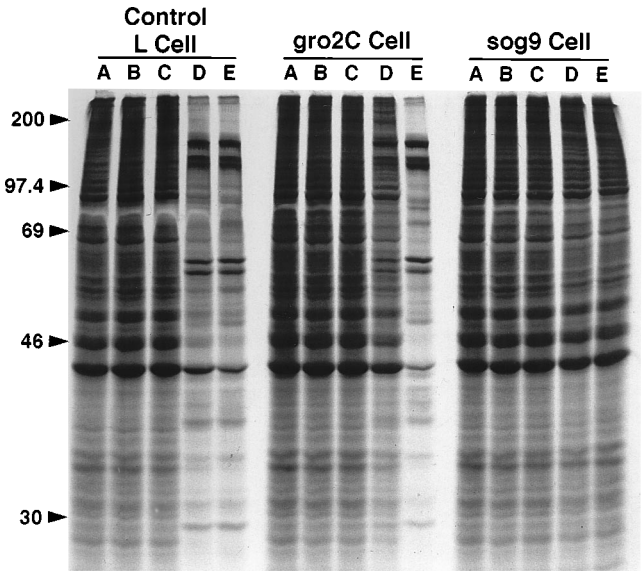

FIG. 3. Polypeptides synthesized by mutant and control L cells after HSV-1 infection. Control L, gro2C, and sog9 cells were infected or mock infected with HSV-1(KOS) at an MOI (determined on control L cells) of 0 (lanes A), 0.03 (lanes B), 0.3 (lanes C), 3 (lanes D), or 30 (lanes E). At 5 h postinfection, monolayers were labeled with [^{35}S]methionine for 1 h. Cell lysates were prepared and analyzed by SDS-PAGE as described in Materials and Methods. The position of molecular mass markers (in kilodaltons) is shown to the left.

tial differences in plaque morphology were noted (Fig. 2). Whereas plaques in gro2C cell monolayers were clear, the sog9 plaques were turbid and were detectable at least 24 h earlier than L or gro2C plaques. This plaque morphology was observed whether neutralizing antibody or agarose overlays were used for plaque assays (data not shown). It can be seen in Fig. 2 that uninfected sog9 cells were only lightly stained with methylene blue, whereas cells associated with plaques stained intensely. The aberrant staining of the cell monolayer could be accounted for by the propensity of methylene blue to stain acidic glycosaminoglycans and suggested that uninfected sog9 cells were relatively deficient in these molecules.

To determine whether the number of plaques that arose in the sog9 cell monolayer corresponded to the number of cells initially infected, two experiments were performed. Cell monolayers were infected with various amounts of HSV-1(KOS) and incubated in the presence of [^{35}S]methionine to determine whether viral gene expression could be detected in sog9 cells (Fig. 3). Viral proteins were detected in control L cells infected at MOIs of 3 and 30 and at an MOI of 30 in the parental gro2C cells. By contrast, no viral gene expression was detected in sog9 cells by autoradiography, and attempts to detect the viral glycoprotein gB on Western immunoblots at the concentrations of virus used in this experiment were also unsuccessful (data not shown). To investigate this further, indirect immunofluorescence was used to detect the HSV-1 immediate-early protein ICP4 at 6 h postinfection (Fig. 4). The number of sog9 cells infected at a given MOI was reduced by nearly 3 orders of magnitude compared with control L cells when differences in input virus were taken into account. Interestingly, the addition of dextran sulfate to the inoculum appeared to increase infection (Fig. 4D, and see below). Thus, plaque assays were an accurate measure of infection efficiency, and the assays suggested that the block to infection of sog9 cells occurred early in the entry pathway prior to the onset of viral gene expression.

Virus adsorption to cell monolayers. To test the attachment proficiency of HSV-1 on sog9 cells, cell monolayers were incubated with radiolabeled virus for 1 h, and then the monolayers were rinsed and the radioactivity associated with adsorbed virus was determined by liquid scintillation spectroscopy (Fig. 5). Substantially less virus adsorbed to sog9 monolayers after 1 h than to either L-cell or gro2C cell monolayers, which suggested that sog9 cells were defective in cell surface molecules to which HSV-1 can attach. However, the reduction in adsorption was less than expected, suggesting that adsorption could not account entirely for the HSV resistance phenotype in these cell lines. Because it was not possible to quantify the extent of functional adsorption in these assays, the contribution of virus adsorption defects to the HSV resistance phenotype of mutant cell lines could not be determined precisely.

Characterization of glycosaminoglycan synthesis. To test whether sog9 cells acquired additional defects in the glycosaminoglycan synthesis pathway compared with the parental gro2C cells, radiolabeled glycosaminoglycans were isolated from cell extracts and analyzed by anion exchange HPLC (Fig. 6). Parental L cells (Fig. 6A) synthesized two major sulfated peaks, representing heparan sulfate (fractions 50 to 55) and chondroitin sulfate (fractions 55 to 70). An additional nonsulfated peak (fractions 45 to 50), representing hyaluronic acid, was also evident. In gro2C cells (Fig. 6B), the profile was essentially the same except for the absence of the heparan sulfate peak. By contrast, sog9 cells (Fig. 6C) had lost the

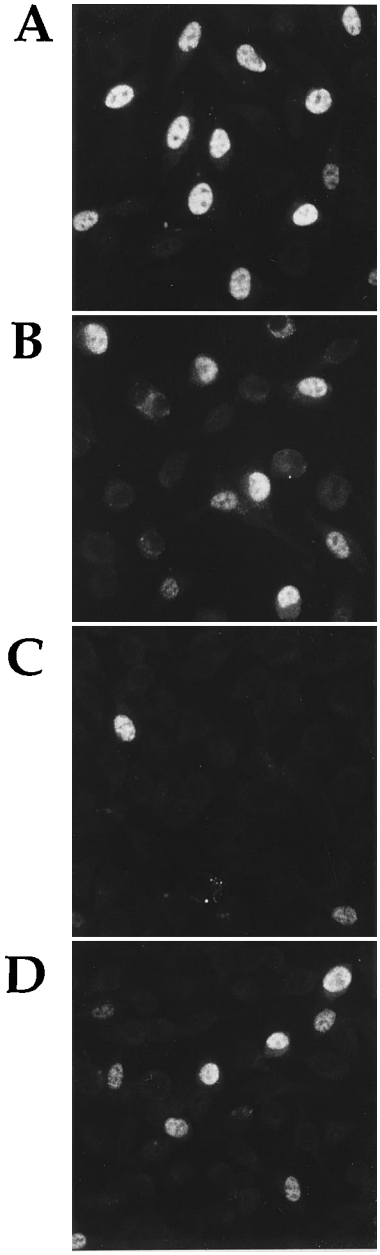

FIG. 4. Indirect immunofluorescence microscopy of HSV-1-infected monolayers. Monolayers of control L cells (A), gro2C cells (B), and sog9 cells (C and D) growing on glass coverslips were infected with HSV-1(KOS) at an MOI (determined on control L cells) of 0.3 (A), 3 (B and D), or 30 (C), in the presence of (D) or absence of (A, B, and C) 300 ng of dextran sulfate per ml. At 5 h postinfection, cells were fixed and ICP4 was detected with anti-ICP4 monoclonal antibody followed by a fluoresceinated second antibody as described in Materials and Methods.

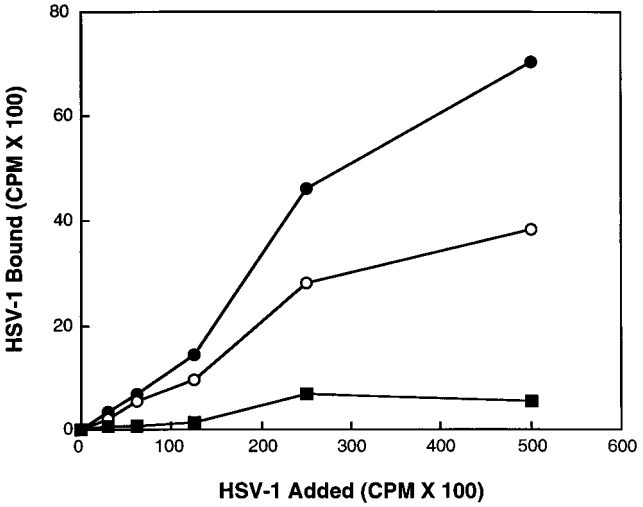

FIG. 5. Adsorption of radiolabeled HSV-1 to mutant and control L-cell monolayers. Confluent monolayers of cells were incubated with increasing concentrations of radiolabeled HSV-1 for 1 h at 0°C. After the adsorption period, monolayers were washed extensively with PBS to remove unbound material. The radioactivity associated with the monolayer was determined by liquid scintillation spectroscopy. Each datum point represents the average of four determinations. Closed circles, L cells; open circles, gro2C cells; closed boxes; sog9 cells.

ability to synthesize any of the major glycosaminoglycan species. Thus, there was a progressive loss of glycosaminoglycan synthetic ability and susceptibility to HSV-1 with each round of selection for HSV resistance.

Effects of polyanions on HSV infection. The perturbation to glycosaminoglycan synthesis in sog9 cells was severe, resulting in a cell surface largely devoid of sulfated glycosaminoglycans. If HSV-1 could not interact with glycosaminoglycans to infect sog9 cells, we reasoned that infection of this cell line would be resistant to further inhibition by soluble heparan sulfate. To test this hypothesis, heparan sulfate was added to the inoculum during HSV-1 infection and its effects were assessed (Fig. 7). As little as 3 μg of heparan sulfate per ml inhibited gro2C cell infection by 90%. This is consistent with previous data showing that HSV-1 infection of gro2C cells is likely mediated by interactions between the virus particle and cell surface chondroitin sulfate glycosaminoglycans, thereby accounting for the ability of soluble heparan sulfate and chondroitin sulfate to inhibit infection (5). By contrast, when sog9 cells were infected in the presence of heparan sulfate, the efficiency of HSV-1 infection was decreased by only 14% at 3 μg of heparan sulfate per ml and by 55% at the highest concentration tested. We also determined that infection of sog9 cells was insensitive to inhibition by chondroitin sulfate or hyaluronic acid (data not shown). Interestingly, gro2C cell infection did not fall below the level of sog9 cell infection under the same conditions (Fig. 7), suggesting that a glycosaminoglycan-independent pathway of infection exists in both cell lines.

To determine whether the interactions of HSV-1 with sog9 cells were principally electrostatic, cells were infected with HSV-1 in the presence of the glycosaminoglycan analog dextran sulfate, which has been shown to be a potent inhibitor of enveloped virus infection (41, 43, 45–47, 55, 64). As shown previously (5), dextran sulfate strongly inhibited the infection of control L cells (Fig. 8A) and had little effect on infection of gro2C cells. By contrast, low concentrations of dextran sulfate actually stimulated HSV-1 infection of sog9 cells by as much as 25-fold. This was also confirmed by indirect immunofluorescence (Fig. 4D). We next determined whether dextran sulfate could stimulate virus adsorption to sog9 cells at 4°C (Fig. 8B). Whereas dextran sulfate exerted little effect on virus adsorption to control L cells and parental gro2C cells, it stimulated adsorption to sog9 cells by nearly fivefold. Because it was not possible to quantify the extent of adventitious binding of radiolabeled virus to cell monolayers, the actual fold stimulation of functional adsorption in the presence of dextran sulfate could not be determined.

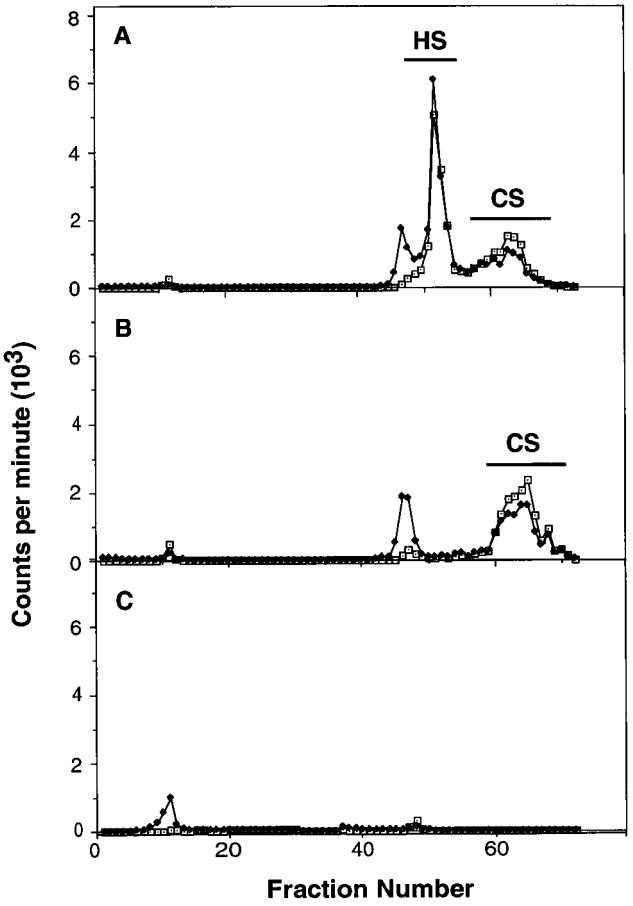

FIG. 6. Glycosaminoglycan synthesis profiles of mutant and control L cells. Monolayers of L, gro2C, and sog9 cells were grown for 3 days in the presence of [^{35}S]sulfate and D-[6-^{3}H]glucosamine. Glycosaminoglycans were isolated and fractionated by HPLC on a TSK DEAE-3SW column as described in Materials and Methods. (A) L cells; (B) gro2C cells; (C) sog9 cells. HS, elution position of heparan sulfate; CS, elution position of chondroitin sulfate. Open squares, [^{35}S]sulfate; closed diamonds, D-[6-^{3}H]glucosamine.

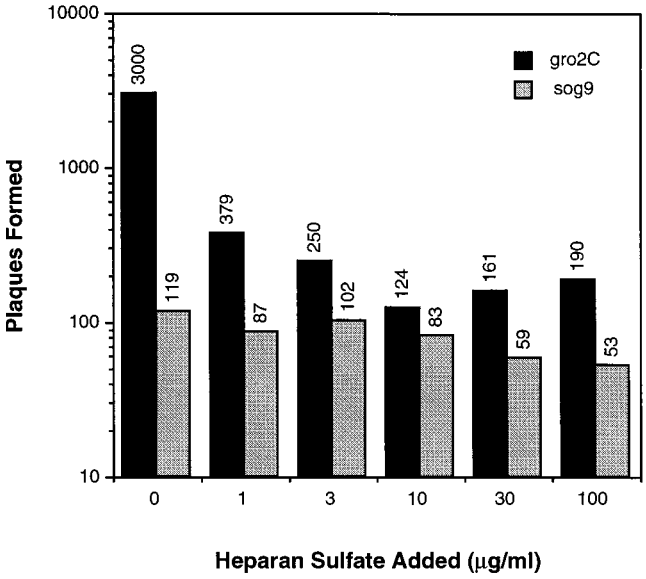

FIG. 7. Effect of soluble heparan sulfate on HSV-1 plaque formation. Monolayers of gro2C and sog9 cells growing in 6-well cluster dishes were inoculated with equivalent amounts of HSV-1 diluted with various concentrations of heparan sulfate. After 1 h of adsorption at 37°C, the inoculum was removed and medium containing 0.1% pooled human IgG was added to the monolayers. The number of plaques in each well was determined after 4 days. Plaque numbers are shown above each bar of the histogram. The experiment was carried out three times and did not vary by more than 10% at any point. The results of a single experiment are shown.

To assess whether dextran sulfate stimulation was mediated by the heparin-binding glycoprotein gC, we repeated the plaque assays with gC-deficient HSV-1(KOS)ΔgC2-3. The relative infection efficiency of the gC-deficient virus on the three cell types was similar to that of wild-type virus (Table 1). Moreover, HSV-1(KOS)ΔgC2-3 infection was stimulated effectively by dextran sulfate, suggesting that gC was not required for dextran sulfate stimulation (Fig. 8C). To test whether the effects were type specific, plaque assays were done with HSV-2(G). Interestingly, there was no stimulation of HSV-2 infection (Fig. 8C). Thus, it appeared that dextran sulfate promoted infection of sog9 cells in a manner that was dependent on both the virus strain and the cell phenotype. Taken together, these data suggest that the block to infection of sog9 cells could be compensated for by soluble glycosaminoglycan analogs present during inoculation.

Because the pathway used by HSV-1 to gain entry to sog9 cells did not involve glycosaminoglycans and was stimulated by dextran sulfate, we investigated whether glycoprotein D, which has been shown to facilitate a late stage of virus attachment, was required for sog9 cell infection. Monolayers of control L and sog9 cells were preincubated and subsequently infected in the presence of soluble gD-1(306t) as described in Materials and Methods. We found that HSV-1 infection was reduced by soluble gD-1(306t) in a concentration-dependent manner. At 500 μg/ml, L-cell infection was reduced by 37%, whereas sog9 cell infection was reduced by 53% (data not shown). These results suggest that gD plays a role in sog9 cell infection.

DISCUSSION

The current hypothesis regarding the role of glycosaminoglycans in promoting HSV infection is that one or more components of the virion, probably gC and gB, interact with glycosaminoglycan moieties to stabilize the virus at the cell surface. Once at the cell surface, the virus engages other components of the host cell which allow it to fuse with the plasma membrane or otherwise enter the cell (11, 21, 22, 42, 48, 51, 62). After infection, the lytic cycle ensues in cultured cells, leading invariably to cell death. We have taken advantage of the lethality of HSV infection to select for cells resistant to infection. Here, we report the stepwise isolation of a cell line, termed sog9, which is highly resistant to HSV-1 infection in part because of the failure of virus adsorption to the cell surface. The most likely explanation for this phenotype is that the cells have lost the ability to express glycosaminoglycans. The gro2C cell line, from which sog9 cells were derived, is defective in heparan sulfate synthesis and is itself approximately 90% resistant to infection compared with control L cells (24). We have shown that infection of gro2C cells is likely mediated by chondroitin sulfate moieties, and infection can be blocked by soluble chondroitin sulfate (5). We reasoned that if the chondroitin sulfate moieties were important for the HSV infection pathway, selecting for HSV-resistant gro2C cells would yield variants that have lost the ability to synthesize these molecules. This hypothesis was confirmed by the isolation of sog9 cells in a single round of selection (Fig. 1 and 6).

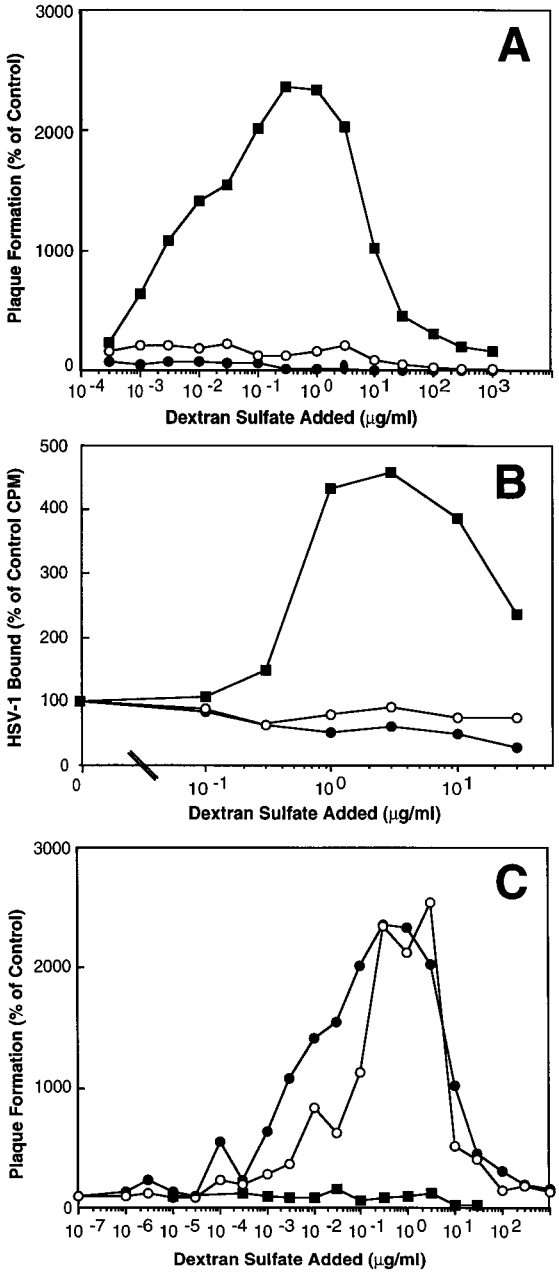

FIG. 8. Effects of soluble dextran sulfate on herpesvirus infection. (A) Effect of soluble dextran sulfate on HSV-1(KOS) plaque formation. Monolayers of L cells, gro2C cells, and sog9 cells were inoculated with HSV-1(KOS) diluted in various concentrations of dextran sulfate. After a 1-h adsorption period, the virus was removed and medium containing 0.1% pooled human IgG was added to the monolayer to allow for plaque formation. Data are expressed as percentages of the infection that occurs in the absence of dextran sulfate. Closed circles, L cells; open circles, gro2C cells; closed squares; sog9 cells. Results are averages of at least three determinations which did not vary by more than approximately 10% at any point. (B) Adsorption of radiolabeled HSV-1 to mutant and control L cells. Radiolabeled HSV-1 (25,000 cpm from the experiment whose results are shown in Fig. 5) diluted in increasing concentrations of dextran sulfate was added to monolayers of L, gro2C, and sog9 cells. After 1 h of adsorption at 4°C, unbound material was washed away and the radioactivity associated with the monolayers was determined by liquid scintillation spectroscopy. Data are expressed as percentages of the radiolabeled virus bound to the monolayer in the absence of dextran sulfate. Closed circles, L cells; open circles, gro2C cells; closed squares, sog9 cells. Results shown are averages of four determinations. (C) Infection of sog9 cells by HSV-1(KOS), HSV-1(KOS)ΔgC2-3, and HSV-2(G) in the presence of dextran sulfate. Monolayers of sog9 cells growing in 6-well-cluster dishes were infected with virus diluted in various concentrations of dextran sulfate. After 1 h of adsorption at 37°C, the virus was removed and medium containing 0.1% pooled human IgG was added to facilitate plaque formation. Data are expressed as percentages of plaque formation that occurs in the absence of dextran sulfate. Closed circles, HSV-1(KOS); open circles, HSV-1(KOS)ΔgC2-3; closed squares, HSV-2(G). Each point shown represents the average of at least two determinations.

We also show that sog9 cells remain susceptible to HSV-1 infection, albeit at a reduced efficiency, which indicates that glycosaminoglycans are not essential for the initiation of HSV infection of mouse L cells (Table 1; Fig. 1 and 4). This result was somewhat surprising because glycosaminoglycans appear to be required for infection of CHO cells (54), suggesting that cell-type-specific or species-specific components play a role in HSV infection. Evidence for the existence of species-specific receptors comes from experiments which show that the M6PR may function as an HSV receptor in primate cells but does not act as a receptor in mouse cells (32). It has also been reported that swine testis cells are relatively resistant to HSV infection despite having a normal complement of cell surface glycosaminoglycans (58). This suggests that a nonglycosaminoglycan HSV receptor is missing from these cells. Our data support the idea that sog9 cells display a second receptor that does not require prior or concurrent interaction with glycosaminoglycans, as evidenced by the observation that soluble heparan sulfate is not an effective inhibitor of HSV infection of sog9 cells (Fig. 7). This result is exactly what is predicted from a model in which soluble heparan sulfate acts as a decoy for interaction with the cell surface and does not otherwise diminish the infectivity of the virus particle itself. In this regard, in the presence of soluble heparan sulfate, gro2C cell infection was not inhibited below the level of sog9 cell infection. This suggests that glycosaminoglycan-dependent and -independent mechanisms of infection can exist side by side. It is interesting that infection of sog9 cells appears to be mediated in part by glycoprotein gD, as it is in normal cells, which suggests that the virus may use a similar mechanism to gain entry to both sog9 and control L cells.

The study of sog9 cells also provides evidence that HSV contains several components capable of interacting with glycosaminoglycans to initiate a productive infection. We have shown that a gC-deficient virus, HSV-1(KOS)ΔgC2-3, has reduced infectivity on sog9 cell monolayers compared with gro2C cells and as such is sensitive to the absence of chondroitin sulfate moieties on the cell surface (Table 1). We have also shown that infection of gro2C cells with HSV-1(KOS)ΔgC2-3 is inhibited effectively by soluble chondroitin sulfate (5). Taken together, these results show that virion components other than gC interact with several cell surface glycosaminoglycans en route to a productive infection. Evidence that gB can serve this function in the absence of gC has recently been reported (25). It is interesting to note that pseudorabies virus (PRV) does not appear to possess multiple glycoproteins which interact with glycosaminoglycans but instead relies on PRV gC to interact with heparan sulfate alone (35). Thus, PRV infects gro2C and sog9 cells with equal efficiency (10% of that with control L cells). Moreover, a gC-deficient PRV strain infects all three cell lines with equal efficiency, indicating that in contrast to the situation with HSV-1, PRV gC appears to be the only glycoprotein which interacts with glycosaminoglycans (35). The differences that we observed for HSV-1 infection of gro2C versus sog9 cells are likely due to defects in glycosaminoglycan expression relevant to HSV-1 infection and not due to a generalized defect which prevents infection by all enveloped viruses.

One of the hallmarks of HSV infection of mutant cells is that

soluble heparan sulfate is unable to substitute for cell surface glycosaminoglycan moieties in the virus infection pathway (24, 54). This suggests that heparan sulfate may play a role in tethering the virus to the cell surface. It has also been demonstrated that heparan sulfate must be present during inoculation to inhibit infection (5). It was therefore curious that HSV-1 infection of sog9 cells was stimulated by extremely low concentrations of the glycosaminoglycan analog dextran sulfate (Fig. 4D and 8). Stimulation by dextran sulfate depended on the virion and the cell insofar as the effect was virus type specific (Fig. 8C) and cell type specific (Fig. 8A and B) and could be mediated by virion components other than the heparin-binding glycoprotein gC (Fig. 8C). Although we do not yet understand the mechanism of action of dextran sulfate, we think it is likely that dextran sulfate promotes a stable interaction of the virus with the host cells because it promotes virus adsorption at 4°C (Fig. 8B). It may be the case that dextran sulfate acts as a bridge between the virus and the sog9 cell surface to enhance infection in the absence of glycosaminoglycans. However, because dextran sulfate stimulates HSV-1 but not HSV-2 infection, this mechanism appears to be different from that used by the virus to contact cell surface glycosaminoglycans, which shows little type specificity. We are not sure why soluble heparan sulfate does not stimulate infection in a similar manner.

It is also possible that postattachment events are impaired in sog9 cells. Our results show that virus attachment efficiency is considerably higher than plaquing efficiency. Because we are not using purified virus, it is possible that some of the virus attachment is nonspecific and as such is nonfunctional. In addition, Shieh and Spear (53) have provided evidence that gB requires heparin (or heparan sulfate) to induce a conformational change necessary for fusogenic function in CHO cells. Although it is possible that fusion is inefficient in sog9 cells, HSV-1(MP) forms syncytial plaques on sog9 monolayers (Table 1), suggesting that fusion in mouse L cells does not share this requirement for heparan sulfate.

One of the strongest results to come from this study is the demonstration that HSV-1 can be used to select stable cell mutants defective in proteoglycan synthesis (pgs). Esko and colleagues have shown previously that CHO cell pgs mutants from five complementation groups could be isolated by screening for cells which fail to synthesize sulfated macromolecules (3, 4, 16–18, 38). The cell lines pgsD 677, which is defective in heparan sulfate biosynthesis, and pgsA 745, defective in both chondroitin sulfate and heparan sulfate synthesis, have been shown to be highly refractory to HSV infection (54). There is no reason to think that the selection protocol used to isolate CHO pgs mutants would preferentially select for HSV-resistant cell lines unless the two phenotypes are linked. Likewise, it is extremely unlikely that the sequential selection protocol that we used to select sog9 cells would yield defects in glycosaminoglycan synthesis unless these molecules were involved in HSV infection. Taken together, these two studies provide strong evidence that the HSV resistance phenotype is a result of the pgs defects in these cell lines.

What could be the defect in sog9 cells? It is clear from the HPLC analysis that sog9 cells have lost the ability to synthesize glycosaminoglycans, including hyaluronic acid (Fig. 6). This could arise if an enzymatic activity common to the synthesis of all glycosaminoglycans was lost. The most likely explanation to account for this phenotype is a reduction in the availability of UDP GlcA, which could be caused by a defect in the formation of UDP GlcA from UDP glucose or in the translocation of UDP GlcA into the Golgi cisternae via the UDP GlcA translocator present in Golgi membranes (28). The analysis of the underlying defect in sog9 cells is currently under investigation.

Several conclusions regarding the infection pathway of HSV can be made from this study. First, glycosaminoglycans do not appear to be essential for infection of mouse L cells. In the absence of glycosaminoglycan synthesis, however, the efficiency of infection is reduced by more than 99%. This suggests that the virus is unable to engage the cell surface effectively in the absence of glycosaminoglycans. A secondary pathway of infection may operate under these conditions. Second, cells expressing only chondroitin sulfate are infected much more efficiently than cells lacking these molecules, suggesting that chondroitin sulfate can play a functional role in the pathway of infection. Third, dextran sulfate stimulates virus adsorption to sog9 cells, leading to a productive infection in a type-specific manner, suggesting that multiple virus components can mediate infection via negatively charged polymers. Finally, this study demonstrates that HSV is a powerful reagent for selection of cells with specific defects in glycosaminoglycan biosynthesis. These cells should be valuable not only for the study of virus entry but also for the investigation of other important biological processes mediated by proteoglycans.

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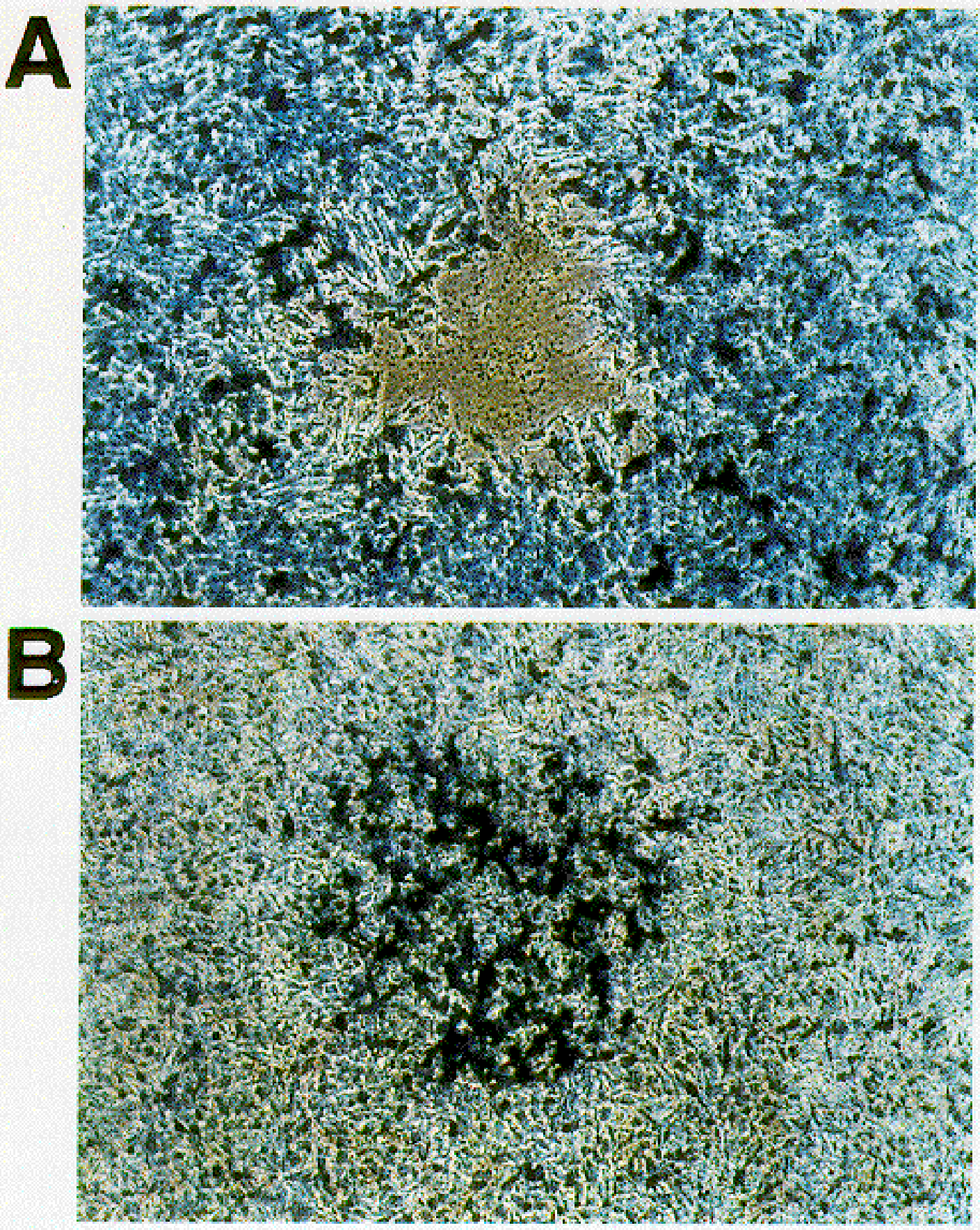
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