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Herpesvirus-Induced Cell Fusion That Is Dependent on Cell Surface Heparan Sulfate or Soluble Heparin

MEI-TSU SHIEH† AND PATRICIA G. SPEAR*

Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611

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The entry of enveloped viruses into animal cells and the cell-to-cell spread of infection via cell fusion require the membrane-fusing activity of viral glycoproteins. This activity can be dependent on variable cell factors or triggered by environmental factors. Here we show that cell fusion induced by herpes simplex virus glycoproteins is dependent on the presence of cell surface glycosaminoglycans, principally heparan sulfate, or on the addition of heparin to the medium. The role of the glycosaminoglycan is probably to alter the conformation of a viral heparin-binding glycoprotein required for the fusion.

For some enveloped viruses, endocytosis may be a prerequisite for the fusion event mediating viral entry into the cell cytoplasm, and cell fusion may not occur unless the infected cells are exposed to medium of low pH. In these cases, the low pH characteristic of endosomes triggers the membrane-fusing activity (3, 23). For other enveloped viruses, endocytosis is not required for viral entry and cell fusion can occur at a physiological pH (3, 23). Membrane-fusing activity (in particular, that leading to cell fusion) may be regulated nonetheless.

In the case of herpes simplex virus (HSV), both cell factors and viral genes influence whether cell fusion occurs after infection (32). For example, infected polykaryocytes are observed in the lesions of HSV-infected individuals, whereas the virus isolates obtained from the lesions usually do not induce the fusion of various cultured cell types, even when the cells are exposed to low pH. This suggests that certain epidermal cells in vivo, but not a variety of cultured cells, may be susceptible to fusion induced by wild-type HSV strains. Viable HSV mutants that induce cell fusion arise during virus propagation in cell culture and are readily identified by plaque morphology (as syncytia instead of clumped rounded cells). These syncytial viral mutants can have genetic alterations in any one of at least four different loci. These loci encode one of the viral glycoproteins required for membrane fusion (gB) and three other viral proteins of unknown function (UL20, UL24, and gK) (32). Thus, viral proteins control the expression of membrane-fusing activity at the cell surface, either by influencing the movement of viral proteins to the cell surface or by influencing their activity. The viral glycoproteins known to be required for HSV-induced cell fusion (and also for viral penetration of the cell) include gB, gD, gH, and gL (4, 10, 21, 22, 26, 27). These glycoproteins form multiple spikes in the virion envelope (33), with gH and gL present as hetero-oligomers (14) and gB as homo-oligomers (28).

In this study we have explored the role of glycosaminoglycans (GAGs), particularly cell surface heparan sulfate and structurally similar heparin, in regulating HSV-induced cell fusion. Two of the HSV glycoproteins, gB and gC, can bind to heparin under physiological conditions (12). The binding of HSV to cells is mediated principally by the interaction of gC with heparan sulfate chains of plasma membrane proteoglycans (12, 29, 34, 37). When gC is absent from the virion, binding with reduced efficiency can occur through the interaction of gB with cell surface heparan sulfate (11). Although gC strongly influences the binding of virus to cells, it is not required for penetration. In contrast, gB is required for penetration and for virus-induced cell fusion, suggesting that the interaction of gB with GAGs could influence gB's activity in inducing membrane fusion.

Chinese hamster ovary (CHO) cells and mutants defective in various aspects of GAG biosynthesis (Table 1) were used to test the role of these sulfated polysaccharides in HSV-induced cell fusion. The two major classes of GAG expressed on wild-type CHO-K1 cells are heparan sulfate and chondroitin sulfate, which differ in patterns of sulfation and in amino sugar and uronic acid compositions (6, 19). Heparin is a more extensively modified and sulfated form of heparan sulfate and is secreted by specialized types of cells (19). The syncytial viral mutant used in this study was HSV-1(MP) (13). Its genetic alterations include a missense mutation in gK that is responsible for the syncytial phenotype (24) and a frameshift mutation resulting in an inability to produce gC (5). Control viruses included HSV-1(mP) (13), a nonsyncytial sibling of HSV-1(MP), and HSV-1(KOS), another nonsyncytial strain.

HSV can infect wild-type CHO-K1 cells, with various degrees of efficiency depending on the viral strain (29), even though the cells do not support a complete replicative cycle. HSV-1(MP) can form syncytial plaques on CHO-K1 cells because of the ability of infected cells to express viral proteins required for the fusion and to recruit adjacent cells into syncytia. HSV-1(MP) cannot form plaques on the heparan sulfate-deficient mutant CHO cells, however, in part because of the greatly reduced binding of HSV to the mutant cells (29). Virus stocks with titers of more than 10^7 PFU/ml on CHO-K1 cells have titers of less than 10 PFU/ml on mutant cells. Because the mutant cells are resistant to HSV infection, we tested whether the mutant cells could be recruited into syncytia induced by infected wild-type cells and whether mutant cells transfected with HSV-1(MP) DNA could form syncytia.

In the first test, CHO-K1 cells were infected with virus at 20 PFU per cell and after 2 h were detached with trypsin-EDTA to inactivate residual virus and to disperse the cells. The infected cells were plated on monolayers of uninfected

* Corresponding author. Mailing address: Northwestern University Medical School, Department of Microbiology-Immunology, Mail Code W213, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-8230. Fax: (312) 503-1339. Electronic mail address: p-spear@nwu.edu.

† Present address: Department of Biological Sciences, Clemson University, Clemson, SC 29634.

TABLE 1. CHO cell lines used[a] and their properties

Cell type	Strain	Biochemical deficiency	Production of GAG		Reference
			Heparan sulfate	Chondroitin sulfate	
Wild type	K1	None	Yes	Yes	
Mutant group					
pgsA	745	Xylosyltransferase	No	No	8
pgsD	677	*N*-Acetylglucosaminyl and glucuronosyltransferases	No	Yes[b]	7, 20
pgsE	606	*N*-Sulfotransferase	Yes[c]	Yes	1, 2

[a] Obtained from J. D. Esko (University of Alabama at Birmingham). Methods for isolation of the mutant cell lines have been described (7).
[b] Chondroitin sulfate accumulates to levels three times higher than in wild-type cells.
[c] The heparan sulfate produced is undersulfated by a factor of two to three.

CHO-K1 cells or pgsA-745 cells in six-well plates. Each well received 8,000 infected cells, because we anticipated about a 5% plating efficiency for the cells. At various times after plating, the monolayers were stained with Giemsa for the quantitation of syncytia. Figure 1 shows that the infected cells were able to induce syncytia on the CHO-K1 cell monolayers much more efficiently, by a factor of 10, than on the pgsA-745 cell monolayers.

In the second test, wild-type and mutant CHO cells were plated on chamber slides in Ham's F12 medium containing 10% fetal bovine serum and, after 24 h, were transfected with viral DNAs from HSV-1(KOS), HSV-1(mP), and HSV-1(MP) (36) at 5 μg per chamber and maintained in the growth medium described above. After 48 h of incubation, the transfected cells were fixed and stained with an anti-ICP4 monoclonal antibody (58S) to identify by immunofluorescence the cells expressing viral genes (29). The fraction of ICP4-positive cells that formed syncytia was determined by counting the nuclei in the fluorescent cells. For CHO-K1 cells, the fraction of ICP4-positive cells that formed syncytia with more than four nuclei was about 25% for HSV-1(MP) DNA and less than 1% for the DNAs of the nonsyncytial virus strains (Fig. 2). The small ICP4-expressing polykaryocytes (two to four nuclei) seen after transfection with the DNAs of all three HSV strains probably resulted mainly from the transfection of preexisting multinucleated cells, which are frequently observed in these cell lines.

Figure 2 also shows that HSV-1(MP) DNA was much less efficient, by a factor of 10, at inducing larger syncytia (more than four nuclei) in the GAG-deficient mutant cells (pgsA-745) than in the wild-type cells. The numbers of smaller polykaryocytes (two to four nuclei) on the two cell types were similar and were somewhat higher than those seen after transfection with the DNAs of the nonsyncytial viruses, indicating that a fraction of the smaller polykaryocytes present on both cell lines might have resulted from HSV-1(MP)-induced syncytium formation. In experiments not shown, the time course of syncytium formation after transfection with HSV-1(MP) DNA was assessed. The fraction of ICP4-positive CHO-K1 cells that formed the larger syncytia (more than four nuclei) was 20% at 24 h and 25% at 48 h after transfection, compared with values of <2% for ICP4-positive pgsA-745 cells at 24 and 48 h.

To determine the effects of the various GAG deficiencies on

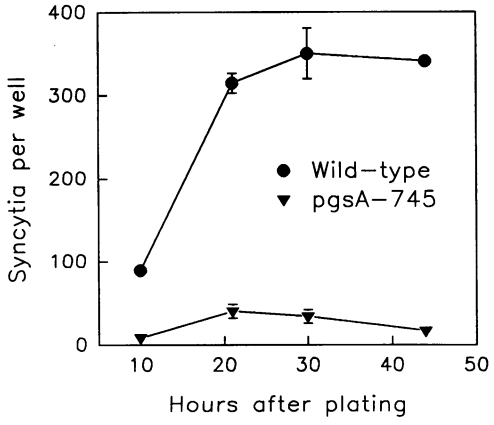

FIG. 1. Syncytium formation by HSV-1(MP)-infected CHO-K1 cells plated on monolayers of wild-type CHO-K1 cells or GAG-deficient pgsA-745 mutant cells. Wild-type CHO-K1 cells were infected with HSV-1(MP), detached for replating on monolayers of CHO-K1 cells or GAG-deficient mutant cells (pgsA-745), and stained for quantitation of syncytia as described in the text. The values shown are the averages of duplicate determinations. The error bars show the range of individual values obtained, except where the range was smaller than the diameter of the symbols.

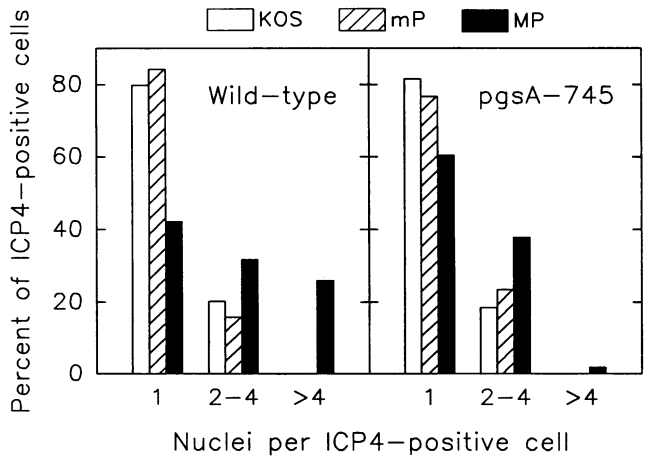

FIG. 2. Syncytium formation after transfection of wild-type CHO-K1 cells and GAG-deficient CHO mutant pgsA-745 cells with the genomic DNAs of HSV-1(KOS), HSV-1(mP), and the fusion-inducing mutant HSV-1(MP). The transfected cells were fixed and stained as described in the text to score the positive cells by microscopy for number of nuclei. For each sample, approximately 150 ICP4-positive cells were scored. In replicate transfection experiments done with HSV-1(MP) DNA, the levels of ICP4-positive cells with more than four nuclei ranged from 19.8 to 27.3% for CHO-K1 cells and from 1.8 to 4.7% for pgsA-745 cells (n = 3). For all experiments, the numbers of ICP4-positive cells per chamber slide ranged from 500 to 1,500.

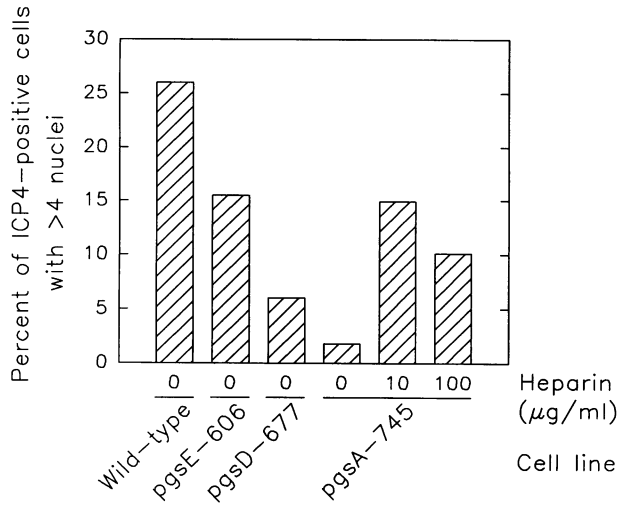

FIG. 3. Syncytium formation after transfection of wild-type CHO-K1 cells and CHO mutant cell lines with HSV-1(MP) DNA. The methods were as described in the text. In replicate determinations of the effects of heparin on pgsA-745 cells transfected with HSV-1(MP) DNA, the levels of ICP4-positive cells with more than four nuclei ranged from 1.8 to 4.7% in the absence of heparin and from 14.9 to 17.9% in the presence of heparin at 10 μg/ml.

HSV-1(MP)-induced syncytium formation, the wild-type and mutant cell lines described in Table 1 were transfected with HSV-1(MP) DNA. For heparin-treated samples, the medium (Ham's F12 medium plus 10% fetal bovine serum) was replaced at 15 h after transfection by the same medium containing heparin (catalog no. H-3125; Sigma) at 10 or 100 μg/ml, and then it was replaced again at 40 h by fresh heparin-containing medium. The cells were fixed and stained at 48 h after transfection, and the numbers of multinucleated cells were determined as described above, except that 300 to 400 ICP4-positive cells were scored. Figure 3 shows that the fraction of ICP4-positive cells in larger syncytia was 26% for wild-type CHO-K1 cells, 15% for pgsE-606 cells, 6% for pgsD-677 cells, and less than 2% for pgsA-745 cells. Therefore, the absence of both heparan sulfate and chondroitin sulfate (pgsA-745) had the largest negative effect on HSV-1(MP)-induced syncytium formation. The absence of heparan sulfate alone (pgsD-677) had a lesser effect, but residual syncytium formation could have been influenced by the enhanced levels of chondroitin sulfate in the mutant cells. While undersulfation of heparan sulfate (pgsE-606) had the least effect, syncytium formation was reduced in parallel with the reduced sulfation.

To determine whether the addition of exogenous soluble heparin could substitute for the absent cell surface GAGs, pgsA-745 cells were transfected with HSV-1(MP) DNA and maintained after transfection in the absence or presence of heparin at 10 or 100 μg/ml. Figures 3 and 4 show that exogenous heparin significantly enhanced the ability of HSV-1(MP) to induce the formation of large syncytia in pgsA-745 cells. In other experiments (data not shown), we tested the effects of heparin at concentrations ranging from 2.5 to 100 μg/ml. Nontransfected CHO cells, wild-type or mutant, were not induced to fuse by heparin. Optimal concentrations for the enhancement of HSV-1(MP)-induced syncytium formation in pgsA-745 cells were between 5 and 10 μg/ml. At these concentrations and higher, heparin had little effect or mildly inhibitory effects on syncytium formation in wild-type CHO-K1 cells.

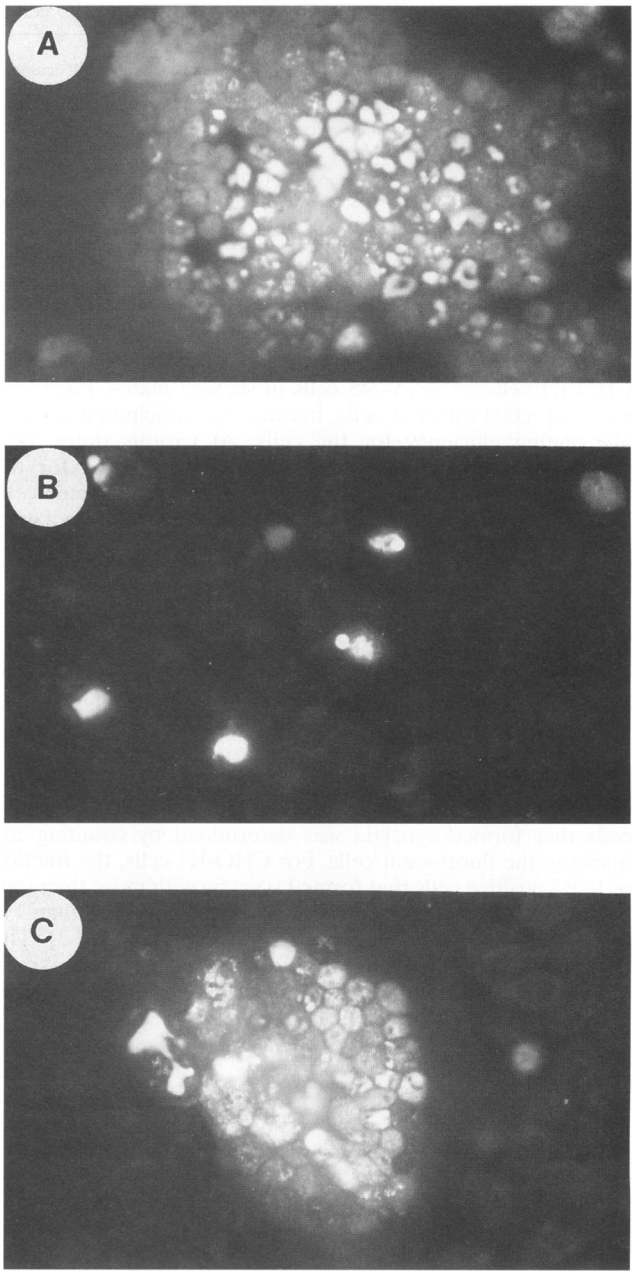

FIG. 4. Representative photographs of syncytia formed on wild-type CHO-K1 cells (A) or on pgsA-745 cells incubated in the presence of heparin (C) after transfection with HSV-1(MP) DNA. (B) Mononucleated and binucleated ICP4-positive cells seen after transfection of pgsA-745 cells with HSV-1(MP) DNA in the absence of heparin. The nuclear fluorescence identifies the transfected cells that express the immediate-early viral protein ICP4. The methods are as described in the legend to Fig. 3.

The effect of heparin shows that the mutant cells are not intrinsically resistant to HSV-induced cell fusion and that they can express the viral proteins required. The mutant cells are also not resistant to fusion induced by other viruses. Wild-type CHO-K1 cells and GAG-deficient mutant pgsA-745 cells were infected with vesicular stomatitis virus (VSV) at 10 PFU per cell and incubated in Dulbecco's modified Eagle medium plus

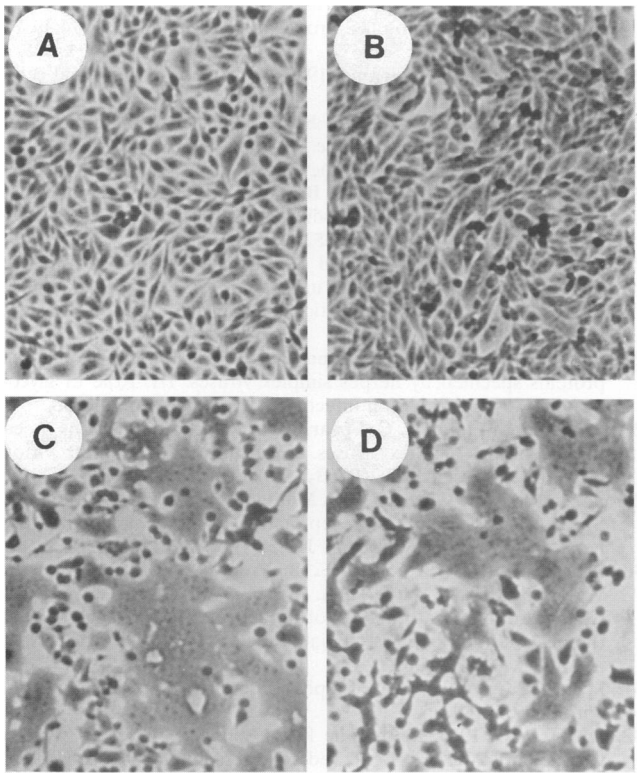

FIG. 5. Syncytium formation after infection of wild-type CHO-K1 cells and GAG-deficient mutant pgsA-745 cells with VSV. (A) Uninfected pgsA-745; (B) uninfected CHO-K1; (C) VSV-infected pgsA-745; (D) VSV-infected CHO-K1.

1% fetal bovine serum for 16 h. The cells were then exposed to a low-pH fusion buffer (1.85 mM $NaH_2PO_4 \cdot H_2O$, 8.39 mM Na_2HPO_4, 2.5 mM NaCl, 10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 10 mM MES [morpholineethanesulfonic acid] [pH 5.5]) for 1 min and then subjected to three washes with phosphate-buffered saline and an additional incubation in medium for 2 h (9). The cells were stained with Giemsa. VSV induced the fusion of CHO-K1 cells and pgsA-745 cells with similar efficiencies (Fig. 5). Fusion of the pgsA-745 cells by VSV did not require the presence of exogenous heparin, indicating that the requirement for cell surface or exogenous GAG is specific to HSV-induced cell fusion.

Cell surface GAGs provide receptors for the binding of HSV to cells (31). Because soluble heparin could not substitute for cell surface GAG in promoting the binding of virus to cells, it was concluded that GAG acts as a true receptor for virus binding and not as a coreceptor that enables the virus to bind to some other cell surface component (29). However, other cell surface molecules are thought to serve as receptors for HSV entry once HSV virions are bound to the cell (16, 17). As recently described for adenovirus (35), it seems likely that the entry of HSV requires one set of receptor-ligand interactions for binding and a second, perhaps overlapping, set of interactions for entry.

The results presented here identify a role for cell surface GAG, principally heparan sulfate, in promoting HSV-induced cell fusion. Because exogenous soluble heparin can substitute for cell surface GAG in promoting the fusion of transfected CHO cells, it seems likely that the role of GAG is not to serve as a receptor for the adhesion of two cells but rather to interact with a heparin-binding protein, thereby promoting fusogenic activity. In numerous cases, interaction of a GAG-binding protein with the appropriate GAG has been shown to greatly enhance or alter the biological activity of the protein (15). The effect of GAG on a protein in the HSV system may be equivalent to the pH-induced alteration in conformation of influenza virus HA and consequent triggering of fusogenic activity (30). A candidate for the molecular target for GAG activation of fusion is HSV gB. HSV-1(MP) expresses gB, which has heparin-binding activity, but not gC, the other known HSV heparin-binding glycoprotein. Moreover, gB but not gC is required for HSV entry (virion-cell fusion) and for syncytium formation (cell-cell fusion). Clearly, proteins other than gB may be required for membrane fusion, and GAG may interact with more than one viral protein and even with a cell surface component. For example, functional binding of fibroblast growth factor to its receptor kinase requires the interaction of a GAG with both the growth factor (25, 38) and the receptor (18).

Cell surface chondroitin sulfate may substitute for heparan sulfate, albeit poorly, in promoting HSV-induced cell fusion, and the degree of sulfation influences the activity of heparan sulfate. It will be important to define the precise structural requirements for GAG in promoting HSV-induced cell fusion. Cells can differ in the kinds of GAG expressed and in the patterns of GAG sulfation (6, 19), and these differences may explain some of the differences in susceptibility of various cells to HSV-induced cell fusion.

In summary, virus-induced membrane fusion is necessary for the replication and cell-to-cell spread of enveloped animal viruses. The membrane-fusing activity can be governed by a variety of factors including viral genes and cell determinants. Among the cell determinants are variations in the pHs of various cell compartments and, as shown here, the expression of cell surface GAGs or the presence of exogenous GAGs. Viruses that resemble HSV in their ability to induce cell fusion at a physiological pH, such as other herpesviruses and the human immunodeficiency virus, may also express fusogenic factors that are influenced by cell surface molecules such as GAGs.

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