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Infection of cells by varicella zoster virus: Inhibition of viral entry by mannose 6-phosphate and heparin

(mannose 6-phosphate receptors/heparin sulfate proteoglycan)

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ABSTRACT **Envelope glycoproteins of varicella zoster virus (VZV) contain mannose 6-phosphate (Man6*P*) residues. We now report that Man6*P* competitively and selectively inhibits infection of cells *in vitro* by cell-free VZV; furthermore, dephosphorylation of VZV by exposure to alkaline phosphatase rapidly destroys infectivity. Cells are also protected from VZV in a concentration-dependent manner by heparin (ED_{50} = 0.23 μg/ml; 95% confidence limits = 0.16–0.26 μg/ml) but not by chondroitin sulfate. Both heparin and Man6*P* are protective only when present about the time of inoculation. Heparin but not Man6*P* interferes with the attachment of VZV to cell surfaces; moreover, VZV binds to heparin-affinity columns. These data are compatible with a working hypothesis, whereby VZV attaches to cell surfaces by binding to a heparan sulfate proteoglycan. This binding stabilizes VZV, making possible a low-affinity interaction with another Man6*P*-dependent receptor, which is necessary for viral entry.**

Varicella zoster virus (VZV) is an α-herpesvirus that causes varicella and zoster (1). Although VZV is extremely contagious *in vivo*, relatively little is known about its entry into target cells. Previous studies have revealed that oligosaccharides derived from glycoproteins of the VZV envelope contain mannose 6-phosphate (Man6*P*; ref. 2); moreover, Man6*P* and other phosphorylated monosaccharides protect cells from the cytopathic effect (CPE) of VZV with an order of potency that parallels their affinity for binding to the cation-independent Man6*P* receptor (MPR^{ci}). Treatment of cells with chloroquine, which reduces the expression of MPR^{ci} at cell surfaces, also protects against infection by VZV. Electron microscopic immunocytochemical observations, furthermore, have revealed that enveloped virions are associated with MPR^{ci} at the cell surface and that newly enveloped virions are incorporated into MPR^{ci}-containing vesicles in the trans Golgi network (3). These observations are compatible with the hypothesis that an interaction of viral glycoproteins with an MPR is important in viral entry. Such a mechanism may also influence the infection of cells by herpes simplex virus (HSV); HSV glycoprotein D (gD) acquires Man6*P* residues and binds to MPRs (4). Binding of either VZV or HSV to an MPR, however, has yet to be demonstrated. Specific cell surface receptors are a critical factor in determining the range of cells that can be infected by a particular virus (5); therefore it is important to determine which receptors mediate infection of cells by VZV.

Viral entry may involve more than a single receptor. Entry of HSV, for example, requires both initial attachment and subsequent internalization (6–8). Different glycoproteins of the HSV envelope have been implicated in each of these steps. Adsorption of HSV is the responsibility of glycoprotein C (gC), and mutant virions from which gC has been deleted are unable to bind to target cells (9, 10). In contrast, antibodies to gD do not inhibit the attachment of virions, but they do prevent the penetration of cells to which the virions have adsorbed (10). If gC and gD each interact with a different receptor, then the cellular protein responsible for adsorption would be different from that which mediates penetration. The cell surface molecules that interact either with gC or gD have not been identified, although gC of HSV-1 (9), like glycoproteins of other herpesviruses (11, 12), binds to heparin-like molecules. Genetic (6) and biochemical (7) studies of mutant CHO cell lines, defective in glycosaminoglycan (GAG) biosynthesis (13, 14), also support the idea that a cell surface heparan sulfate proteoglycan (HSPG) is required for the attachment and subsequent infection of cells by HSV. It is plausible that the uptake of VZV, like that of HSV, involves more than a single step and more than one type of receptor. The current experiments were undertaken to test the hypotheses that adsorption of VZV is mediated by HSPG and that a receptor for which Man6*P* is a ligand plays a subsequent role in viral entry.

MATERIALS AND METHODS

Confluent monolayers of human embryonic lung fibroblasts (HELFs) and cell-free VZV were prepared as described (2). To determine the stage of viral infection affected by Man6*P*, HELF monolayers were exposed to cell-free VZV [>25 plaque-forming units (pfu)/ml]. Man6*P* (20 mM final concentration) was added at various intervals following inoculation. Cultures were then incubated for 3 or 4 days, after which plaques were counted. To analyze the effect of phosphorylated monosaccharides on the expression of gp1 on the surfaces of infected cells, HELF monolayers were infected with ≈50 pfu of cell-free VZV in medium containing Man6*P* (10–20 mM) or glucose 1-phosphate (Glc1*P*; 10–20 mM). Neither monosaccharide was added to controls. HELFs were cultured for 3 or 4 days and VZV gp1 was quantified by RIA. For this assay, infected cells were exposed to a murine monoclonal antibody to VZV gp1 for 30 min. After being washed, the cells were incubated with rabbit anti-mouse antibodies and then with ^{125}I-labeled protein A. The cells were washed and solubilized, and their radioactivity was quantified by liquid scintillation. To determine the effect on infectivity of exposure of VZV to alkaline phosphatase, preparations of cell-free VZV were incubated in medium containing *Escherichia coli* alkaline phosphatase (0.3–1.0 unit/ml) for 2–60 min. Control preparations were similarly incubated but without the enzyme.

The method for ligand blot analysis using soluble MPR^{ci} as a probe was that of Valenzano *et al.* (15). Proteins from infected and control cells were extracted, separated by

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Abbreviations: VZV, varicella zoster virus; HELF, human embryonic lung fibroblasts; HSV, herpes simplex virus; HSPG, heparin sulfate proteoglycan; GAG, glycosaminoglycan; pfu, plaque-forming units; MPR, Man6*P* receptor; CPE, cytopathic effect.
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SDS/8% PAGE, blotted onto nitrocellulose, blocked with 3% nonfat dry milk solids, and incubated for 2 h with biotinylated, soluble MPR^{ci} (provided by Peter Lobel; Center for Advanced Biotechnology and Medicine, Piscataway, NJ). Bound MPR^{ci} was detected with horseradish peroxidase-conjugated avidin.

Heparin and chondroitin sulfate (Sigma) were utilized to study the effect of GAGs on the infection of HELFs by VZV. HELF monolayers were exposed to 25–50 pfu of cell-free VZV in medium that did or did not (controls) contain these molecules and incubated for 2 h at 37°C. After being washed, the cultures were maintained for 4 days, and plaques were counted. To study the effect of phosphorylated monosaccharides and GAGs on cell surface binding of VZV, HELF monolayers were exposed to 25–50 pfu of cell-free VZV at 4°C for 1 h in medium containing heparin (8 μg/ml), chondroitin sulfate (8 μg/ml), Man6*P* (20 mM), or Glc1*P* (20 mM). The cells were washed with cold medium, and the amount of gp1 on the cell surface was measured by RIA. VZV binding to affinity columns containing Sepharose CL-4B beads conjugated with heparin was assessed by adding cell-free VZV diluted with maintenance medium to the columns at room temperature. Control columns contained only supporting elements or unconjugated beads. The column flow-through and wash buffer were collected and added to HELF monolayers. Plaques were counted after 4 days. The effects of GAGs and phosphorylated monosaccharides on the attachment of VZV to the surfaces of HELF cells were evaluated by Southern analysis of dot blots (16). HELF monolayers were exposed to 25–50 pfu of cell-free VZV for 1 h. The cells were lysed in buffer (10.0 mM Tris·HCl, pH 8.0/1.0 mM EDTA/250 mM NaCl/0.2% SDS/100 μg of proteinase K per ml) at 50°C for 4 h. DNA was extracted with phenol/chloroform, precipitated with ethanol, air dried, and resuspended in buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA). DNA probes prepared by random priming were labeled with [^{32}P]dCTP (New England Nuclear). Dot blots of the extracted DNA were prepared on 1% agarose gels (0.04 M Tris acetate/0.01 M EDTA). DNA was denatured in alkaline buffer, nicked with UV light, transferred to nitrocellulose, washed briefly, and baked at 80°C for 1 h. The blots were prehybridized (43°C for 6 h) and hybridized (43°C overnight with 1.5×10^7 cpm of the ^{32}P-labeled cDNA probe) in buffer [5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% SDS/100 μg of denatured salmon sperm DNA per ml]. Hybridized blots were washed (twice in 2× SSC/0.1% SDS; once in 0.2× SSC/0.1% SDS). Blots were then exposed to film for 1 h at −70°C with an intensifying screen.

RESULTS

Man6*P* Antagonizes Infection of Cells by VZV and Acts at an Early Stage in the Infective Process. To determine whether Man6*P* simply masks CPE or whether it actually prevents infection, we used an RIA to detect gp1 on cell surfaces. Intact cells were exposed to anti-gp1 and bound antibody was detected with ^{125}I-labeled protein A. The amount of radioactivity (in cpm) bound to cells was proportional to the amount of VZV in the inoculum; moreover, the cpm correlated well with estimates of infection obtained by quantifying CPE (Fig. 1 *A* and *B*). Expression of viral protein as a result of VZV infection can thus be quantified by RIA of gp1. Man6*P* was as effective in preventing expression of gp1 as in suppressing CPE (Fig. 1*C*). These data confirm that Man6*P* protects cells from infection by VZV.

To determine when Man6*P* inhibits infection, Man6*P* (20 mM) was added at various times from 0 to 480 min following inoculation of HELF cells with VZV. Man6*P* afforded significant protection ($P < 0.01$) when added within 0–30 min of exposure to the virus but not when added after 120 min (Fig. 1*D*). These observations indicate that Man6*P* blocks an early event in the infection of cells by VZV.

To determine whether VZV binds to MPR^{ci}, VZV-related antigens from infected cells were separated by SDS/PAGE, blotted onto nitrocellulose, and probed with a biotinylated soluble form of the MPR^{ci}. This assay has been used to demonstrate the binding of acid hydrolases to MPR^{ci} (15). Immunoblotting with antibodies to gp1 confirmed that VZV antigens were present. The soluble MPR^{ci} bound to several bands on the blots; however, there were no MPR^{ci}-labeled

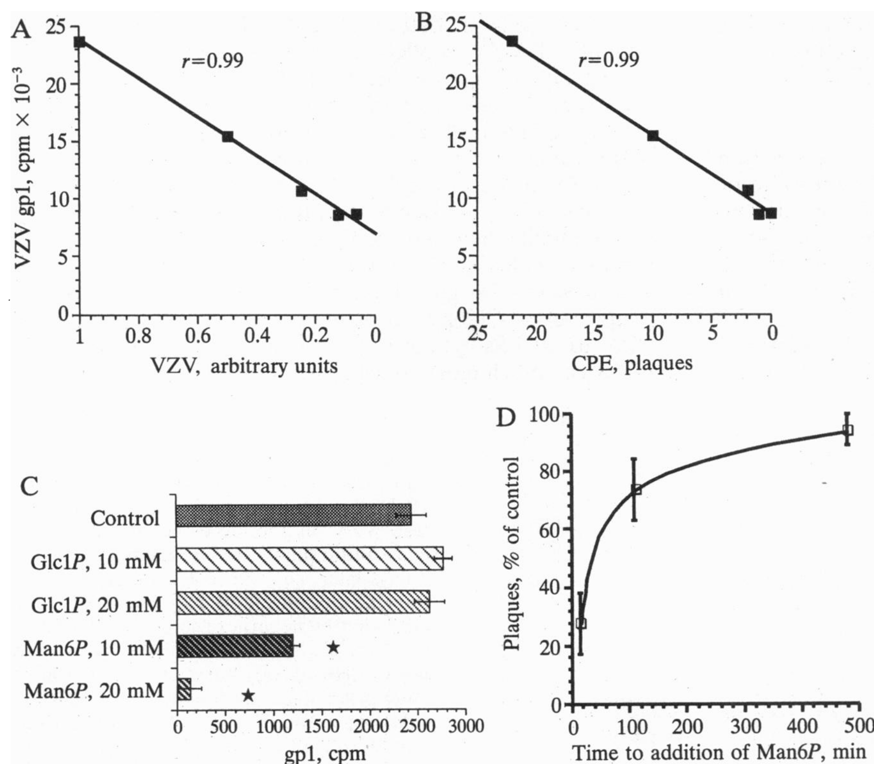

FIG. 1. Man6*P* acts early in the infective process to protect HELF cells from infection by VZV. (*A* and *B*) Expression of gp1 on cell surfaces measured by RIA quantifies infection of cells by VZV; CPE (*B*) correlates well with surface expression of gp1 (*A*). (*C*) Man6*P* but not Glc1*P* inhibits expression of gp1. ★, Significantly different from control. (*D*) Extent of infection is plotted as a function of the time between the inoculation of cultures with VZV and the addition of Man6*P* (20 mM). Man6*P* antagonizes infectivity only if it is added shortly after inoculation.

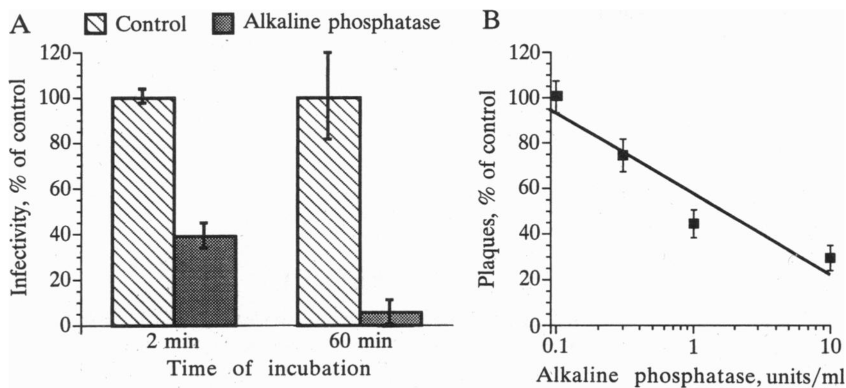

FIG. 2. Dephosphorylation of cell-free VZV by exposure to alkaline phosphatase rapidly destroys infectivity. (*A*) Alkaline phosphatase inhibits infectivity of VZV within 2 min. Incubation of VZV in control medium at the same pH for the same period of time has no effect. (*B*) Effect of alkaline phosphatase on viral infectivity is concentration dependent.

bands in extracts from VZV-infected cells that were not also seen in extracts from uninfected cells (data not shown). As direct evidence for binding of VZV to the MPR^{ci} was not obtained, we tested the role of phosphorylated glycoproteins of the viral envelope in infection. Cell-free VZV was treated with alkaline phosphatase, which has been shown to dephosphorylate Man6*P* residues of VZV glycoproteins and lysosomal enzymes (2, 17). Alkaline phosphatase reduced the infectivity of VZV within 2 min (Fig. 2*A*); this effect was concentration dependent (Fig. 2*B*). In contrast, incubation of the virus in control medium had no effect on infectivity. These observations are consistent with the idea that removal of Man6*P* from VZV envelope glycoproteins impairs infectivity.

Heparin Protects Cells from Infection by VZV. Heparin is known to inhibit the infection of cells by several viruses (7, 11, 18, 19). The effect of heparin on the infection of HELFs by VZV was tested by adding heparin or, as a control, chondroitin sulfate (0.01–10 μg/ml) to the medium at the same time as an inoculum containing cell-free VZV. Heparin (Fig. 3 *A* and *B*) but not chondroitin sulfate (Fig. 3*B*) was a potent and concentration-dependent antagonist of VZV infection; protection was a logarithmic function of the heparin concentration (r = 0.95; $P < 0.001$). The ED_{50} for heparin was 0.23 μg/ml (95% confidence limits = 0.16–0.26 μg/ml), and at 10 μg/ml, infectivity was almost eliminated ($P < 0.001$). In contrast, chondroitin sulfate did not affect VZV infectivity ($r = 0.115$).

Heparin Acts at an Early Stage in the Infective Process to Prevent Infection of Cells by VZV. If heparin blocks the adhesion of VZV to cell surfaces, then it should protect cells only when it is present around the time of inoculation and not after VZV has begun to penetrate target cells. This prediction was tested by comparing protection conferred by heparin or chondroitin sulfate added at 0 or 90 min after inoculation. Heparin protected against VZV only at 0 min (Fig. 4*A*); when added 90 min after inoculation, heparin was no longer effective (Fig. 4*B*). Chondroitin sulfate did not protect HELFs from VZV at either time (Fig. 4 *A* and *B*).

Heparin Blocks the Attachment of VZV to Cell Surfaces at 4°C. The early action of heparin and Man6*P* would be explained if these agents either blocked the attachment of VZV to cell surfaces or interfered with the subsequent process of internalization. To distinguish these possibilities, HELFs were inoculated with VZV at 4°C (to prevent endocytosis) in the presence of heparin, chondroitin sulfate, Man6*P*, or Glc1*P*. The virions were allowed to adsorb for 1 h before the cells were washed. VZV binding to cells was then estimated by measuring the concentration of gp1 by RIA. Identically treated cultures were maintained for 4 days at 37°C to assay CPE. Heparin but not chondroitin sulfate, Man6*P*, or Glc1*P* prevented attachment of VZV to cells at 4°C (Fig. 5*A*). In contrast, both Man6*P* and heparin specifically reduced CPE; infectivity was not affected by chondroitin sulfate or Glc1*P*. The ability of heparin to prevent the association of VZV with host cells was studied at physiological temperature (37°C), when both binding and endocytosis of virions can occur. Cell-free VZV was absorbed to HELF cells for 60 min. Cell-associated DNA encoding gp1 was quantified by Southern analysis of dot blots (Fig. 5*B*). The amount of hybridizing gp1 DNA was diminished by heparin but not by Man6*P* or Glc1*P*. Thus, heparin, but not Man6*P*, prevents the association of VZV with target cells under physiological conditions.

Intact Virions Bind to Heparin. Cell-free VZV was applied in equal aliquots to a column packed with either Sepharose or heparin-Sepharose. The column eluates were added to cultures of HELF cells to determine CPE. The infectivity of the aliquots of VZV applied to each column was assessed by adding equal aliquots directly to HELF cells and used to normalize the infectivity of the column eluates. The heparin-affinity column dramatically reduced infectivity (Fig. 6*A*; $P <$ 0.01 vs. control; $n = 6$). VZV thus binds to heparin. The idea that VZV binds to a cell surface HSPG was also tested by culturing HELF for 12 h in the presence of chlorate (10 μM), which inhibits the sulfation of GAGs. Cell-free VZV (50 pfu) was then applied to the cultures for 60 min. Adhesion of VZV was measured by gp1 RIA, and infectivity was assessed by plaque assay. Chlorate treatment reduced the adherence of VZV to HELFs by ≈60% ($P < 0.001$; $n = 4$) and inhibited infectivity much more strikingly (Fig. 6*B*; $P < 0.001$; $n = 16$).

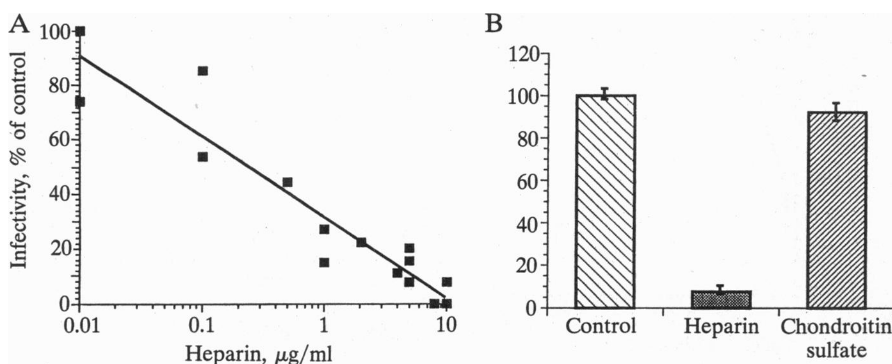

FIG. 3. Heparin but not chondroitin sulfate protects cells from the CPE of VZV. HELF monolayers were inoculated with cell-free VZV in the presence of heparin or chondroitin sulfate, and CPE was determined 4 days later. (*A*) Concentration-effect curve showing the concentration dependence of the inhibition by heparin of infection of HELFs by VZV. (*B*) Number of plaques found when heparin (10 μg/ml) was present in the inoculum is less than that found in controls ($P <$ 0.001) or in the presence of chondroitin sulfate (10 μg/ml; $P < 0.001$). Control, $n = 6$; heparin, $n = 6$; chondroitin sulfate, $n = 14$.

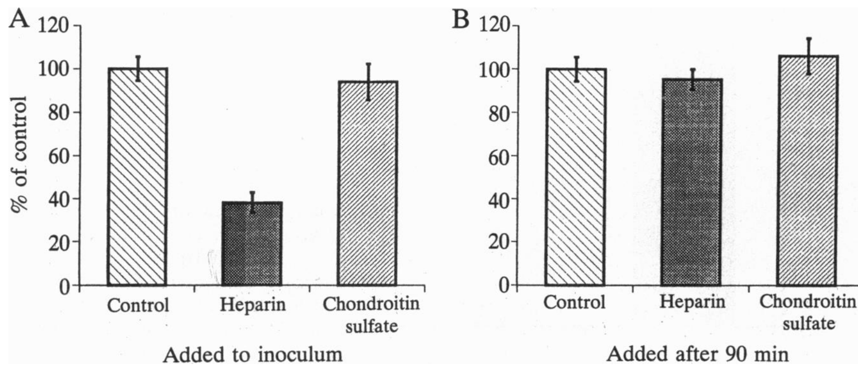

FIG. 4. Heparin interferes with an early event in the infective process. HELF monolayers were exposed to cell-free VZV for 3 h. Heparin or chondroitin sulfate (10 μg/ml) was either included in the inoculum or added 90 min after VZV. Plaques were counted 4 days after the cells were exposed to VZV. Heparin antagonized infection when present in the inoculum ($P < 0.001$ vs. control or vs. chondroitin sulfate) but failed to reduce infectivity when added 90 min after VZV. Each column represents the mean value of eight cultures.

DISCUSSION

Previous studies showed that Man6*P* decreases CPE when cells are inoculated with cell-free VZV (2); however, these investigations did not determine whether Man6*P* prevents or simply masks the visible manifestation of infection. The RIA used in the current study to quantify the cell surface expression of gp1 showed that expression of gp1 correlated well with CPE and that both were decreased in a concentration-dependent manner by Man6*P*. These data confirm that Man6*P* interferes with the infection of cells by VZV. The ability of Man6*P* to protect cells from infection by VZV, moreover, occurred at an early stage in the infective process, indicating that Man6*P* could either interfere with the attachment of the virions or with their subsequent penetration of the plasma membrane. Since the plasma membrane is not permeable to Man6*P* (20), it is unlikely that it could act at a site that is not accessible to the extracellular medium. Such sites include the cell surface or the interiors or endosomes, to which Man6*P* and virions could gain entrance by endocytosis. Since Man6*P* failed to block the binding of VZV to cell surfaces at 4°C, it thus seems likely that Man6*P* inhibits a step that follows the initial attachment of the virions, such as the penetration of the plasma membrane by VZV.

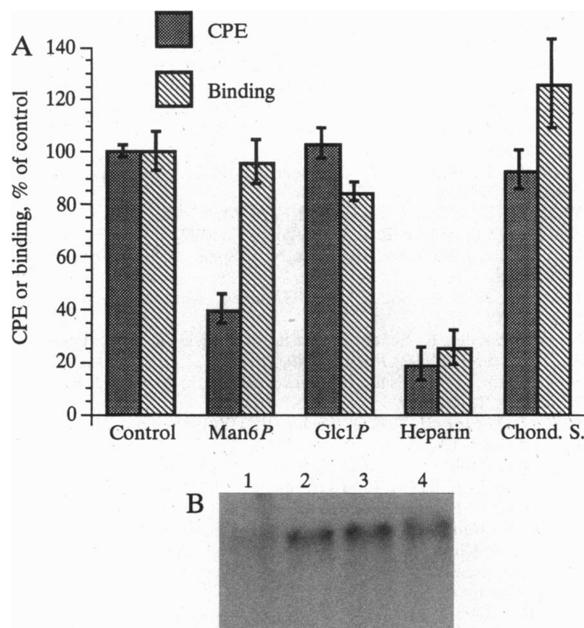

FIG. 5. Heparin and Man6*P* protect HELFs from infection by VZV, but only heparin prevents binding of VZV to cell surfaces at 4°C or its association with cells at 37°C. (*A*) Cell-free VZV was adsorbed onto HELF monolayers at 4°C in the presence or absence (control) of Man6*P* (20 mM), Glc1*P* (20 mM), heparin (8 μg/ml), or chondroitin sulfate (Chond. S.; 8 μg/ml) and gp1 binding was determined by RIA. Infectivity (CPE) was assessed in similarly treated cultures. For Man6*P*, the difference from control in CPE is significant ($P < 0.001$). For heparin, the differences from control in both CPE and bound gp1 are significant ($P < 0.001$). None of the other values are significantly different from the corresponding control. For gp1 binding, each column represents the mean of nine cultures, except chondroitin sulfate, where $n = 3$. For CPE, the numbers of cultures were as follows: control = 26; Man6*P* = 16; Glc1*P* = 16; heparin = 11; chondroitin sulfate = 3. (*B*) Heparin but not Man6*P* inhibits the association of VZV with HELFs at 37°C. HELF monolayers were exposed to cell-free VZV for 60 min in the presence or absence (control; lane 4) of heparin (lane 1), Man6*P* (lane 2), or Glc1*P* (lane 3). The blots were probed with ^{32}P-labeled gp1 cDNA. The amount of DNA encoding gp1 is diminished in extracts of cells exposed to VZV in the presence of heparin but not Man6*P* or Glc1*P*.

Exogenous Man6*P* may disrupt an interaction between VZV and an MPR. MPR-mediated events, however, are not the only cell surface interactions that can be inhibited by Man6*P* (21). Man6*P* also antagonizes natural killer cell-mediated cytotoxicity (22), which is independent of MPRs (23); nevertheless, in contrast to such MPR-independent effects, for which the effectiveness of Man6*P* is shared by a broad range of phosphorylated oligosaccharides (21, 23), the rank order of potency of oligosaccharides in providing protection from VZV parallels their affinity for MPRs (2). This specificity and the protective effect of chloroquine, which interferes with the cycling of free MPRs to the plasmalemma, support the idea that an MPR plays a role in infection. While this evidence is indirect, the current observation that the infectivity of cell-free VZV is inhibited with remarkable speed by alkaline phosphatase suggests that the Man6*P* residues of the glycoproteins of the VZV envelope are essential for infection of cells by VZV. HSV has also been reported to be sensitive to alkaline phosphatase (24). The site affected by Man6*P* could be located on the cell surface or within endosomes. Lysosomal enzymes taken up by endosomes, however, have been shown to be dephosphorylated (25). The similar dephosphorylation of VZV would probably prevent infection of cells by means of endosomes.

Our data are consistent with the idea that a cellular site that recognizes Man6*P* is involved in the penetration of cells by VZV, but there is no direct evidence that glycoproteins of the viral envelope actually bind to the MPR^{ci}. We could not demonstrate the binding of a soluble MPR^{ci} (15) to viral antigens by ligand blotting, even though similar blots detect the binding of the soluble MPR^{ci} to lysosomal enzymes (15). Technical reasons could account for our failure to detect the binding of MPR^{ci} to viral products in ligand blots. First, the binding of viral glycoproteins to the MPR^{ci} might be conformationally specific and thus not detectable on blots in which the antigens are denatured. Protein determinants are known to impair the binding of lysosomal enzymes, such as cathepsin L, to the MPR^{ci} (26). Second, VZV might bind to the cation-dependent MPR, MPR^{cd}, for which no probe is available, rather than to the MPR^{ci}. Third, since VZV characteristically infects only a relatively small proportion of cells in culture, the binding of the MPR^{ci} to a viral protein might be masked by the relatively much greater binding of the MPR^{ci} to normal cellular

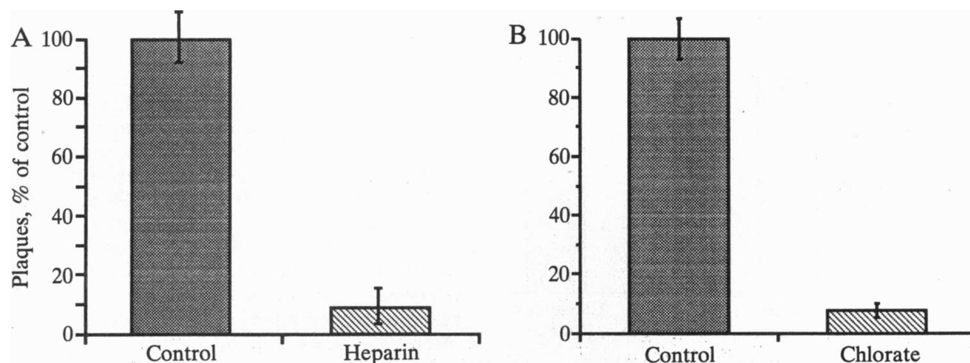

FIG. 6. VZV binds to heparin. (*A*) Cell-free VZV was applied to columns containing either heparin-conjugated or control beads. Infectious viral particles in the flow-through were quantified by plaque assay. (*B*) Treatment of HELFs with chlorate inhibits the infectivity of VZV.

proteins, such as lysosomal enzymes. Finally, the affinity of viral glycoproteins for an MPR may be too low for the physical interaction between the two to be detected in this assay. Intact virions may be polyvalent ligands and thus bind more effectively to receptors than oligosaccharides, which may be monovalent. If the affinity of an MPR for VZV is low, then before it can interact with an MPR, VZV would probably have to be stabilized by binding first to another receptor. Binding of VZV to HSPG could serve this purpose.

That heparin protects target cells from HSV was reported almost 3 decades ago (27), but the inhibition was thought to be nonspecific. More recently, heparin or other sulfated polysaccharides have been shown to selectively inhibit the infectivity of many viruses, including HSV-1 and -2 (7, 18), cytomegalovirus (11, 19), vesicular stomatitis virus (18), pseudorabies virus (12), and human immunodeficiency virus (18, 28–30). Heparin probably inhibits infection by interfering with the binding of virions to HSPG on cell surfaces. Cell surface receptors that bind HSV have now been shown to be HSPG (6). Although the attachment of both HSV-1 and -2 to cells is clearly dependent on an HSPG, there are also additional type-specific receptors on plasma membranes (31), implying that more than a single receptor is involved in the infection of cells by HSV. Binding of virions to an HSPG may be a necessary prerequisite for the interaction of viral particles with another molecule that also participates in the penetration of cells. In fact, different envelope glycoproteins mediate the attachment (9) and penetration (32) of HSV, so different receptors are probably involved in the infection process. Similar two-receptor models have been proposed for the interactions of nonviral ligands with the plasma membrane. For example, the high-affinity binding and biological activity of basic fibroblast growth factor (bFGF) require that bFGF interact with both HSPG and FGF receptors (33, 34). Furthermore, adenovirus internalization but not attachment is promoted by the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (5).

We found that heparin inhibited the infection of HELFs by VZV in a concentration-dependent manner, with a potency comparable to that of its ability to prevent infection of cells by HSV (6, 7). As with Man6*P*, the protective effect of heparin was exerted early in the infection process, consistent with the idea that heparin interferes with viral attachment to, or penetration of, target cells. In contrast to Man6*P*, heparin inhibited binding of VZV to cell surfaces at 4°C and its association with cells at 37°C; moreover, VZV bound to heparin. Antagonism by chlorate of proteoglycan sulfation inhibited both the adhesion of VZV to cell surfaces and infection, supporting the idea that VZV binds to cell surface HSPG. The specificity of heparin's effect suggests that the interaction of HSPG with VZV is a structural recognition event.

A heuristic model based on the current data that explains the penetration of target cells by VZV is as follows: VZV first attaches to cells by binding to an HSPG. This binding stabilizes the virions and enables them to interact with a cellular site that recognizes Man6*P*. This binding is probably weak, which is why prior HSPG stabilization is needed for the Man6*P*-dependent process to be effective. When presented by HSPG binding to the Man6*P*-dependent site, the latter receptor participates in mediating viral penetration. Evidence that the Man6*P*-dependent site is an MPR exists but remains indirect. The advantage of the two-step model for viral entry, involving sequential interactions with HSPG and a Man6*P*-dependent site, is that it can be tested.

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