JOURNAL OF VIROLOGY, June 1995, p. 3517–3528 Vol. 69, No. 6
0022-538X/95/$04.00+0

Role of Mannose-6-Phosphate Receptors in Herpes Simplex Virus Entry into Cells and Cell-to-Cell Transmission

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Received 3 November 1994/Accepted 21 February 1995

Herpes simplex virus (HSV) glycoprotein D (gD) is essential for virus entry into cells, is modified with mannose-6-phosphate (M-6-P), and binds to both the 275-kDa M-6-P receptor (MPR) and the 46-kDa MPR (C. R. Brunetti, R. L. Burke, S. Kornfeld, W. Gregory, K. S. Dingwell, F. Masiarz, and D. C. Johnson, J. Biol. Chem. 269:17067–17074, 1994). Since MPRs are found on the surfaces of mammalian cells, we tested the hypothesis that MPRs could serve as receptors for HSV during virus entry into cells. A soluble form of the 275-kDa MPR, derived from fetal bovine serum, inhibited HSV plaques on monkey Vero cells, as did polyclonal rabbit anti-MPR antibodies. In addition, the number and size of HSV plaques were reduced when cells were treated with bovine serum albumin conjugated with pentamannose-phosphate (PM-PO_4-BSA), a bulky ligand which can serve as a high-affinity ligand for MPRs. These data imply that HSV can use MPRs to enter cells; however, other molecules must also serve as receptors for HSV because a reasonable fraction of virus could enter cells treated with even the highest concentrations of these inhibitors. Consistent with the possibility that there are other receptors, HSV produced the same number of plaques on MPR-deficient mouse fibroblasts as were produced on normal mouse fibroblasts, but there was no inhibition with PM-PO_4-BSA with either of these embryonic mouse cells. Together, these results demonstrate that HSV does not rely solely on MPRs to enter cells, although MPRs apparently play some role in virus entry into some cell types and, perhaps, act as one of a number of cell surface molecules that can facilitate entry. We also found that HSV produced small plaques on human fibroblasts derived from patients with pseudo-Hurler's polydystrophy, cells in which glycoproteins are not modified with M-6-P residues and yet production of infectious HSV particles was not altered in the pseudo-Hurler cells. In addition, HSV plaque size was reduced by PM-PO_4-BSA; therefore, it appears that M-6-P residues and MPRs are required for efficient transmission of HSV between cells, a process which differs in some respects from entry of exogenous virus particles.

Viral receptors are molecules which facilitate binding of viruses to the surface of cells and entry into the cells. Ideally, if a virus is dependent on specific receptors for penetration into host cells, antibodies or ligands which bind to the receptor should inhibit virus infection. Additionally, cell lines which lack the putative receptor should be resistant to infection, and if these cells are made to express the receptor, e.g., by transfection, the cells can then be infected by the virus. However, these optimum criteria are frequently not satisfied, and there is growing evidence that many animal viruses utilize multiple pathways to enter host cells. For example, reoviruses enter different cell types by using distinct receptors (9, 11), and human immunodeficiency virus (HIV) appears to be able to enter cells by a CD4-dependent mechanism (42) as well as CD4-independent mechanisms (19, 25, 58). There are also viruses that use multiple receptor molecules sequentially to enter cells. For example, adenovirus type 2 (Ad2) utilizes fiber proteins, which extend from penton bases at the icosahedral vertices of the viral capsid, to attach to or adsorb onto cells (14, 46), whereas penton bases promote internalization by interacting with vitronectin integrins (62). Antibodies specific for the vitronectin receptor reduced Ad2 internalization but not viral attachment, and ligands for vitronectin integrins inhibited Ad2 entry, although the inhibition was often not complete, suggesting that other molecules may also promote entry of Ad2 (62).

Herpes simplex viruses (HSVs) can infect a very broad range of cell types in vivo and in vitro, including fibroblasts, keratinocytes, epithelial cells, glial cells, and neurons of rodent, rabbit, monkey, or human origin, although infection of rodent cells is frequently less efficient (48). Therefore, cellular receptors for HSV must be ubiquitous or HSV utilizes multiple receptors or entry pathways with different cells. HSV encodes at least 12 membrane glycoproteins, 4 of which, gB, gD, gH, and gL, are required for entry of the virus into all cultured cells that have been tested to date (6, 20, 38, 49); however, there is mounting evidence that other glycoproteins may be involved in HSV entry into diverse cell types, especially more highly differentiated cells. For example, gE and gI play a role in cell-to-cell transmission of HSV across junctions formed between human fibroblasts (1a, 15) and between neurons (14a). In addition, there is evidence for multiple HSV receptors on polarized epithelial cells (50). Therefore, it is likely that HSV uses multiple and cell-specific receptors for different cell types.

There is also good evidence that HSV uses distinct cell surface receptors in a sequential fashion to enter cells. Initially the virus adsorbs onto heparan sulfate glycosaminoglycans (GAGs) (51, 64) and certain chondroitin sulfate GAGs (36), which are very numerous components of the extracellular matrix and plasma membrane. This interaction appears to be mediated primarily by gC (27) and may serve to concentrate the virus on the cell surface or activate the virus in some

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manner so that subsequent interactions can occur. There is evidence that following the initial adsorption step, HSV interacts with receptors which are required for virus entry and which are much more limited in number than the sites to which the virus can adsorb. Addison et al. (1) and later Johnson and Ligas (31) found that cells treated with approximately 5,000 UV-inactivated virus particles per cell were resistant to infection with HSV. The inhibition of virus entry by UV-inactivated HSV particles was mediated at the level of virus entry and not at postentry stages of replication, e.g., transport to the nucleus or early transcription, because UV-inactivated HSV added to cells after adsorption of infectious HSV at 4°C failed to inhibit synthesis of early HSV proteins, yet both types of virus particles could enter cells (31). In contrast to virus particles containing gD, UV-inactivated virus particles lacking gD failed to prevent entry of infectious HSV type 1 (HSV-1) or HSV-2, suggesting that gD is required in order that virus particles can interact with this limited set of cell surface receptors required for virus entry. Supporting this hypothesis, cell lines constitutively expressing relatively high levels of gD have been found to be resistant to infection by HSV (7, 32). Presumably, gD produced by the cell sequesters a cellular protein required for viral entry, although mutations which overcome the interference phenotype can alter viral gD, and thus other explanations have been proposed (8, 13). In addition, soluble forms of HSV-1 and HSV-2 gD were able to bind to the cell surface in a saturable manner, the binding was dependent on cell surface proteins, and soluble gD inhibited HSV entry into cells without affecting virus adsorption (30). Together, these results provide compelling evidence that HSV gD acts to engage a set of cell surface receptors which are relatively restricted in number and which facilitate virus entry into cells at a stage subsequent to virus adsorption onto more numerous cell surface GAGs. Whether any of the other HSV proteins which are required for virus entry act as receptor-binding proteins is not clear; however, there is evidence that HSV uses distinct receptors to enter different cell types (50).

Recently we demonstrated that HSV gD bound to both the 275-kDa cation-independent mannose-6-phosphate (M-6-P) receptor (CI-MPR) and the 46-kDa cation-dependent M-6-P receptor (CD-MPR) and was modified with M-6-P (5). Both M-6-P receptors (MPRs) sort glycoproteins modified with M-6-P to lysosomes by binding the lysosomal enzymes in the trans-Golgi compartment and diverting the enzymes into the endosomal pathway (reviewed in reference 12). MPRs are also found on the surfaces of cells, almost exclusively in clathrin-coated pits, and there the receptors also bind extracellular lysosomal enzymes and direct them to endosomes and lysosomes (reviewed in references 34 and 35). A single pool of MPRs cycle continuously between the Golgi, endosomes, and the plasma membrane. The cell surface 275-kDa CI-MPR also serves as a receptor for insulin-like growth factor II (IGF-II) (41, 44).

In this study, we investigated the role of MPRs in the entry of HSV into cultured cells and in cell-to-cell spread of the virus. A synthetic ligand or antibodies which bind to MPRs and a soluble form of the 275-kDa CI-MPR inhibited HSV entry and production of HSV plaques on monkey cells. However, mouse cells lacking both the 46-kDa CD-MPR and the 275-kDa CI-MPR could be infected by HSV. Therefore, MPRs are not absolutely required for HSV entry into cells but may represent one of several pathways by which HSV enters cells. We also obtained evidence that M-6-P modification of HSV glycoproteins play a role in cell-to-cell spread of the virus by using human fibroblasts incapable of phosphorylating mannose residues.

MATERIALS AND METHODS

Cells. Monkey Vero cells were grown in α minimal essential medium (α-MEM; GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 7% fetal bovine serum (FBS; Boknek, Mississauga, Ontario, Canada). Human skin fibroblasts derived from a healthy person (GM0080) or a pseudo-Hurler patient (GM3391) were obtained from the Human Genetic Mutant Cell Repository, Camden, N.J., and the cells were grown in α-MEM supplemented with 20% FBS. Human epithelial tonsil cells were grown in Dulbecco's modified minimal essential medium (D-MEM) supplemented with 10% FBS. Mouse fibroblasts were derived from embryos of wild-type (BALB/c) mice or from mice lacking the 275-kDa CI-MPR or both the 275-kDa CI-MPR and the 46-kDa CD-MPR 13.5 days after gestation, and the cells were propagated in D-MEM supplemented with 20% FBS as previously described (39, 40).

Viruses. Wild-type HSV-1 strain F, obtained from P. G. Spear (then at the University of Chicago), and wild-type HSV-2 strain 333 (33) (obtained from B. Roizman, University of Chicago) and the HSV-1 mutant QAA, which lacks all three N-linked glycosylation sites in gD (53), obtained from G. H. Cohen and R. J. Eisenberg (University of Pennsylvania, Philadelphia), were all propagated and titered on Vero cell monolayers. The HSV-1 gD-negative mutants F-US6kan and F-gDβ, which is also gI negative, were propagated and titered on VD60 cells which express gD (38). Vesicular stomatitis virus (VSV) strain Indiana was a generous gift from L. Prevec (McMaster University, Hamilton, Ontario, Canada).

Reagents and antibodies. Thiophosgene, bovine serum albumin (BSA), M-6-P, and glucose-6-phosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). [^{35}S]methionine and [^{35}S]cysteine were obtained from New England Nuclear (Mississauga, Ontario, Canada). Soluble gD-2t, which lacks the transmembrane domain and cytoplasmic tail of HSV-2 gD, was secreted and purified from the media of recombinant CHO cells (2, 3, 5a) and was produced at Chiron Corp., Emeryville, Calif. Human gamma globulin (HGG), a source of human neutralizing anti-HSV antibodies, was obtained from the Canadian Red Cross. Rabbit antiserum specific for the VSV strain Indiana N protein was a gift from L. Prevec (McMaster University). Rabbit antisera which recognizes HSV thymidine kinase was a kind gift from W. Summers (Yale University, New Haven, Conn.).

Preparation of PM-PO_4, PM-PO_4-BSA, and PM-PO_4-Sepharose. Pentamannose-phosphate (PM-PO_4) was prepared as described by Slodki et al. (52) from yeast *Pichia* (*Hansenula*) *holstii* phosphomannan, which was kindly supplied by M. E. Slodki, U.S. Department of Agriculture, Peoria, Ill. PM-PO_4 was modified with [aminophenyl]-ethylamine (APEA) by the procedure described by Jeffrey et al. (29). A solution of PM-PO_4 in water (300 to 400 mg/5 ml) was mixed with an equal volume of APEA and incubated overnight, then sodium borohydride (125 mg) was added, and the mixture was incubated for 3 h at room temperature. The mixture was further diluted with 4 to 5 ml of water, and the pH was adjusted to between 5 and 6 by using glacial acetic acid. The material was applied to a column of Sephadex G-10 equilibrated in 50 mM sodium borate (pH 8.3), and fractions containing the peak amounts of PM-PO_4-APEA were pooled. To prepare PM-PO_4 conjugated to BSA (PM-PO_4-BSA), PM-PO_4-APEA (15 to 20 mg/4 ml) was layered over 2.5 ml of chloroform containing 7.5 μl of thiophosgene and stirred at room temperature for 3 h. The aqueous layer was transferred to a glass centrifuge tube and extracted three times with chloroform, the chloroform was removed by bubbling a stream of N_2 through the solution, then 1.5 ml of 0.3 M NaCl–0.05 M sodium borate (pH 9.5) containing 3 mg of BSA was added, and the solution was incubated overnight at room temperature. The reaction mixture was applied to a Sephadex G-50 column, and the PM-PO_4-BSA was collected in the void volume. PM-PO_4 was coupled to CNBr-Sepharose by mixing 200 to 300 mg of PM-PO_4-APEA with 50 ml of CNBr-Sepharose (Pharmacia) for 2 days at 4°C. PM-PO_4-Sepharose was then incubated with 0.2 M ethanolamine (pH 8.0) for 2 h at room temperature and then washed successively with 0.1 M Tris-HCl (pH 9.0) containing 2 M NaCl and 0.1 M sodium acetate (pH 3.5) containing 2 M NaCl.

Purification of the soluble form of the 275-kDa MPR (225-kDa MPR) from FBS by using PM-PO_4-Sepharose. FBS was dialyzed against 50 mM imadazole-HCl (pH 6.5)–150 mM NaCl–5 mM EDTA, then diluted with an equal volume of this buffer, and applied to the PM-PO_4-Sepharose column as described by Hoflack and Kornfeld (28). IGF-II was stripped from the soluble 225-kDa MPR by washing the column with 10 mM glucose-6-phosphate (32a). The column was washed exhaustively with 50 mM imadazole-HCl (pH 6.5)–150 mM NaCl, and the soluble 225-kDa MPR was eluted in 50 mM imadazole (pH 6.5)–150 mM NaCl–6 mM M-6-P.

Purification of the 275- and 46-kDa MPRs from bovine testes. The 275- and 46-kDa MPRs were purified from bovine testis by PM-PO_4-Sepharose as previously described (37). Briefly, 400 g of bovine testis was homogenized for 1 min in a Waring blender, and the pellet was resuspended in 800 ml of 0.1 N acetic acid–0.1 M NaH_2PO_4 and centrifuged for 15 min at 10,000 × *g*. The pellet was resuspended in 3 liters of 0.4 M KCl–20 mM imadazole (pH 7.0)–1% Triton X-100, mixed for 60 min at 4°C, and centrifuged at 22,000 × *g* for 60 min. The pH was adjusted to 6.5 with 2 N acetic acid and immediately applied to a PM-PO_4-Sepharose column. The column was washed with 50 mM imadazole-HCl (pH 6.5)–150 mM NaCl–5 mM $MnCl_2$–0.02% NaN_3–0.05% Triton X-100 (usually 12 to 18 h), then washed with 50 mM imadazole-HCl–150 mM NaCl–5 mM $MnCl_2$–1 mg of octylglucoside per ml, and eluted with this same buffer containing 5 or 6 mM M-6-P.

Production of antibodies directed to the 275- and 46-kDa MPRs. Approxi-

mately 150 μg of both the 275- and 46-kDa MPRs purified from bovine testis (see Fig. 2A) was mixed with 1.5 ml of Freund's complete adjuvant and injected into three 6-week-old New Zealand White rabbits by intramuscular and subcutaneous routes. After 30, 60, and 90 days, each rabbit was boosted with approximately 300 μg of purified MPRs in 2 ml of MF59-100, an oil-based adjuvant containing 5% squalene, 0.5% Tween 80, 0.5% Span-85, and 100 μg of muramyl tripeptide-phosphoethanolamine per ml. The final injection consisted of 1.8 mg of purified MPRs in 1 ml of MF59-100, and 10 days later the rabbits were bled. Similarly, each rabbit was injected initially with gD-2t (100 μg) in Freund's complete adjuvant and subsequently with 50 μg in MF59-100 adjuvant. Rabbit immunoglobulin G (IgG) was prepared from the pooled sera by repeated NH_4SO_2 precipitation followed by exhaustive dialysis against 20 mM Tris-HCl (pH 7.2) and chromatography on DEAE-Affigel Blue (Bio-Rad, Mississauga, Ontario, Canada) by procedures described by the manufacturer, which resulted in a preparation which consisted predominantly of IgG as assessed by polyacrylamide gel electrophoresis and Coomassie blue staining. Following chromatography, the purified IgG was dialyzed against α-MEM and concentrated by using a Centricon-30 membrane (Amicon, Beverly, Mass.).

Inhibition of HSV-2 plaque production by soluble MPR. Soluble bovine 225-kDa MPR was dialyzed extensively against D-MEM, diluted with D-MEM containing 1% FBS, and mixed with extracellular HSV-2(333) (obtained from the culture supernatant of infected human embryo tonsil cells) for 30 min at 37°C. These mixtures were added to Vero cells or mouse embryonic fibroblasts growing in 12- or 24-well dishes (approximately 1×10^5 to 2×10^5 cells per well) for 90 min at 37°C. The virus and soluble 225-kDa MPR were removed, and D-MEM containing the appropriate concentrations of soluble 225-kDa MPR, 1% FBS, and 0.2% HGG was added. The plaques were allowed to develop for 24 h, the cells were stained with crystal violet, and plaques were counted.

Inhibition of HSV-2 plaque production by IgG purified from anti-MPR sera. Vero cell monolayers growing in 12-well dishes (approximately 3×10^5 to 4×10^5 cells per well) were treated for 30 min at 32°C with IgG purified from anti-gD sera, anti-MPR sera, or preimmune sera diluted in α-MEM containing 1% FBS. Approximately 150 PFU of HSV-2(333) per well was added, and the cells were incubated for an additional 90 min at 37°C. The medium was removed, and cells were treated with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) for 1 min at room temperature. The cells were washed two times with α-MEM and incubated for 36 h with α-MEM containing 1% FBS, 0.2% HGG, and an appropriate amount of antiserum. Plaques were stained with crystal violet approximately 36 h postinfection.

Inhibition of HSV plaque production by PM-PO_4-BSA. Vero cell monolayers in 12-well dishes (3×10^5 to 4×10^5 cells per well) were incubated in the presence of 0, 0.3, 0.6, 1.1, 2.3, or 4.5 μM PM-PO_4-BSA in 225 μl α-MEM containing 1% FBS for 40 min at 37°C. The cells were infected with either HSV-1(F) derived from the culture supernatant of Vero cells or a cell culture supernatant containing VSV, and the cells were incubated for an additional 60 min at 37°C. Viruses and PM-PO_4-BSA were removed, and the cells washed two times with α-MEM containing 1% FBS. HSV-infected cells were resuspended in α-MEM containing 1% FBS, 0.2% HGG, and the appropriate concentrations of PM-PO_4-BSA, and after 36 h, the cells were stained with crystal violet and plaques were counted. Cells infected with VSV were overlaid with α-MEM containing 1% FBS, 1% low-melting-point agar (ICN, St. Laurent, Quebec, Canada), and an appropriate concentration of PM-PO_4-BSA; after 12 to 18 h, the wells were overlaid with phosphate-buffered saline (PBS) containing 1% neutral red and incubated for 2 h at 37°C before plaques were counted.

Radiolabeling of cells, immunoprecipitation, and gel electrophoresis. Approximately 10^6 mouse fibroblasts derived from wild-type, 275-kDa CI-MPR-deficient, or 46-kDa CD-MPR and 275-kDa CI-MPR (46/275-kDa MPR)-deficient mouse embryos were infected with either HSV-1(F) or VSV at 1 PFU per cell. Two hours after infection, the cells were washed, incubated with medium lacking cysteine and methionine and supplemented with [^{35}S]methionine and [^{35}S]cysteine (100 μCi of each per ml), and incubated for an additional 3 h. Alternatively, Vero cells treated or not treated with PM-PO_4-BSA were infected with HSV-1(F) or VSV and after 3 h incubated with medium lacking methionine and supplemented with [^{35}S]methionine (100 μCi/ml) for 2 h. Cell extracts were produced by lysing cells in Nonidet P-40 (NP-40)–sodium deoxycholate (DOC) buffer (1% NP-40, 0.5% DOC, 50 mM Tris-HCl [pH 7.5], 100 mM NaCl) containing 2 mg of BSA per ml and 1 mM phenymethylsulfonyl fluoride and stored overnight at −70°C. The extracts were thawed and clarified by centrifugation at 16,000 × *g* for 30 min and then mixed with anti-HSV thymidine kinase or anti-VSV N serum (5 μl of serum per extract from 3×10^5 cells) for 60 min. Protein A-Sepharose beads (Pharmacia, Baie d'Urfé, Quebec, Canada) were added, and the samples were incubated for an additional 2 h. The protein A-Sepharose beads were washed three times with NP-40–DOC buffer and then resuspended in 50 mM Tris (pH 6.8)–2% sodium dodecyl sulfate (SDS)–10% glycerol–bromophenol blue–2% β-mercaptoethanol. Samples were boiled for 5 min to elute the proteins, which were loaded onto 10% *N*, *N*′-diallyltartardiamide cross-linked polyacrylamide gels. The gels were dried and exposed to Kodak XAR film.

Detection of the 46- and 275-kDa MPRs by Western immunoblotting. A preparation containing approximately 0.2 μg of the 46- and 275-kDa MPRs from bovine testes was resolved on 7.5% polyacrylamide gels and transferred to nitrocellulose as previously described (5). The nitrocellulose membranes were incubated for 6 h with PBS–1% skim milk–0.5% BSA and then for 8 h with PBS–0.5% fish gelatin (Sigma)–0.5% BSA (PGB buffer). The blots were incubated for 1 h with anti-MPR serum in PGB buffer, washed three times with PGB buffer, and then probed with [^{125}I]protein A (Dupont, Mississauga, Ontario, Canada) for 1 h. The nitrocellulose was washed three times with PGB buffer, and the proteins were visualized with PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, Calif.).

HSV replication on pseudo-Hurler cells. Human primary fibroblast GM0080 and GM3391 monolayers in 12-well dishes (approximately 10^5 cells per well) were infected with wild-type HSV-1(F) or HSV-1 mutant QAA, F-US6kan, or F-gDβ (approximately 150 PFU per well) diluted in α-MEM containing 1% FBS. Three hours after infection, the virus inoculum was removed and α-MEM containing 1% FBS and 0.2% HGG was added. Fifty-six hours after infection, the cells were stained with crystal violet, and plaques counted and photographed. For single-step growth analysis, pseudo-Hurler (GM3391) or normal (GM0080) fibroblast monolayers growing in 12-well dishes (approximately 10^5 cells per well) were infected with HSV-1(F) at 10 PFU per cell in α-MEM containing 1% FBS. Medium and unadsorbed virus were removed 2 h later, and 1 ml of α-MEM containing 1% FBS was added. At various times after infection, the cells were scraped into the medium and sonicated, and infectious HSV was assayed by plaque production, using monolayers of Vero cells.

RESULTS

A soluble form of the 275-kDa MPR inhibits HSV-2 plaque production. To investigate the role of MPRs in entry of HSV into cells, we attempted to block infectivity of HSV by incubating the virus with a soluble form of the 275-kDa MPR. FBS contains a soluble form of the 275-kDa MPR (and not the 46-kDa MPR) which is produced by proteolytic release of the extracytoplasmic domain of the receptor and has an apparent molecular mass of 225 kDa (10); this MPR is designated the 225-kDa MPR. The precise cleavage site has not been defined, but the soluble MPR binds both M-6-P-modified proteins and IGF-II. The 225-kDa MPR was purified from FBS by PM-PO_4 affinity chromatography (16, 37). PM-PO_4 affinity chromatography yields a high degree of purification of either the 225-kDa MPR or the 275- and 46-kDa membrane-associated MPRs (28, 37, 49a). This high degree of purity (>94%) (49a) produced in a single step is related to the fact that columns can be washed extensively and then eluted in the same buffer with the addition of 5 or 6 mM M-6-P. Contaminants (e.g., bound by ionic interactions) are not specifically eluted under these conditions. After SDS-polyacrylamide gel electrophoresis of the MPR preparations from FBS, a major protein species of approximately 225 kDa was detected by Coomassie blue staining (Fig. 1, inset). We also observed a minor protein band migrating slightly faster than the 225-kDa soluble MPR, which is probably a different form of the 225-kDa MPR, because soluble gD-2t interacted with this protein on ligand blots (not shown).

Preparations of HSV-2 derived from cell culture supernatants were mixed with soluble MPR (0 to 6 μM, corresponding to 0 to 1.5 mg/ml) and then the HSV-2–protein mixture was plated on Vero cell monolayers for 90 min. After this incubation, the cells were washed and incubated with the appropriate concentrations of the soluble 225-kDa MPR for a further 24 h; then the cell monolayers were stained, and viral plaques were counted. There were reductions in the numbers of HSV-2 plaques produced on Vero cell monolayers with increasing concentrations of soluble 225-kDa MPR, but there was no inhibition with similar concentrations of BSA (Fig. 1). In this experiment, 6 μM 225-kDa MPR inhibited HSV-2 plaques by greater than 75%. When soluble 225-kDa MPR was present only during the first 90 min of the infection, reductions in the number of plaques were not observed (Fig. 1). Because of the availability of limited amounts of the soluble MPR, and because we found it necessary to incubate cells both before and after virus adsorption, these experiments were not performed with replicate wells. Instead, numerous experiments using different preparations of the protein were performed in the same

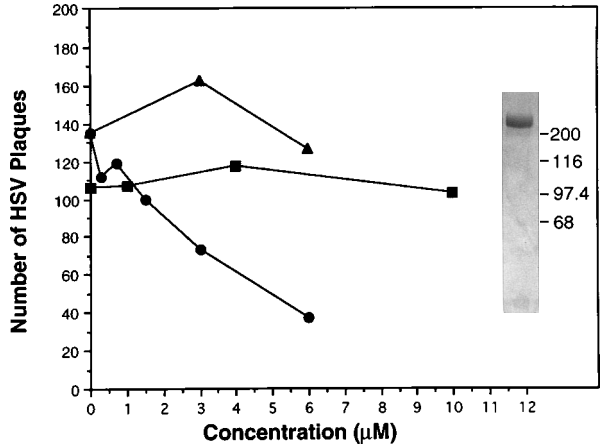

FIG. 1. Inhibition of HSV-2 plaques by soluble MPR. HSV-2(333) was incubated for 30 min at 37°C in medium containing 1% FBS and various concentrations of purified soluble MPR or BSA. The virus-protein mixtures were then incubated with Vero cells in 24-well dishes for 90 min at 37°C, the virus-protein mixtures were removed, and the cells were washed with medium. The cells were then incubated for 24 h with α-MEM containing 1% FBS (▲) or α-MEM containing 1% FBS and either soluble MPR (●) or BSA (■) before the monolayers were stained with crystal violet and plaques were counted. The inset shows a Coomassie blue-stained profile of the soluble MPR purified from FBS; sizes are indicated in kilodaltons.

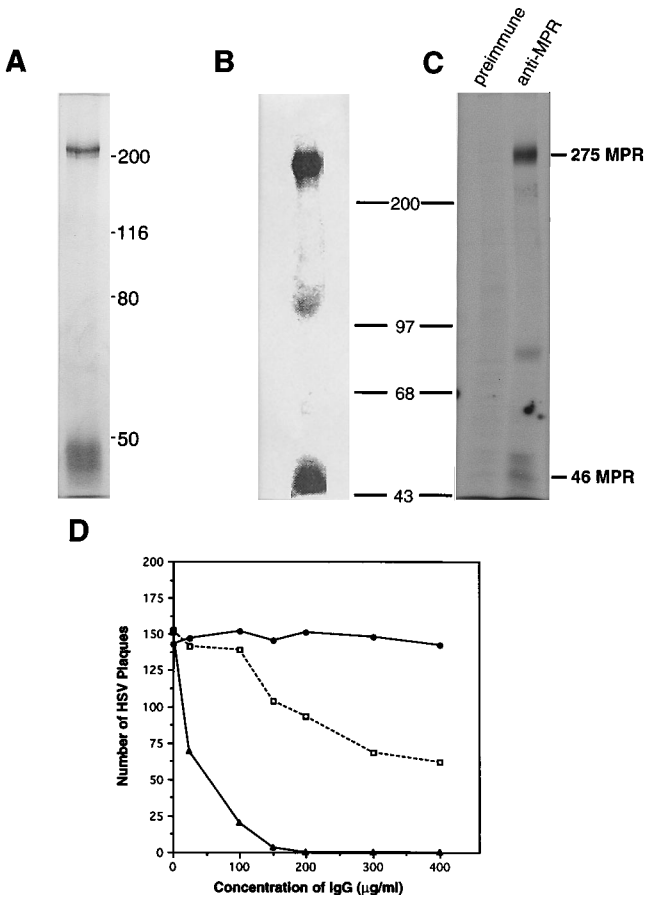

FIG. 2. Inhibition of HSV-2 plaques by anti-MPR antibodies. (A) A preparation of 275- and 46-kDa MPRs used to produce the anti-MPR sera was separated on a 7.5% polyacrylamide gel, and proteins were visualized by staining with Coomassie brilliant blue. (B) Western blot demonstrating that anti-MPR antibodies recognize both the 275- and 46-kDa MPRs. The 275- and 46-kDa MPRs purified from bovine testes were subjected to electrophoresis on a 7.5% polyacrylamide gel, proteins were transferred to nitrocelluose, and then blots were incubated with anti-MPR antiserum diluted 1:200, washed, and incubated with ^{125}I-labeled protein A. Proteins were visualized with a PhosphorImager. (C) Immunoprecipitation using anti-MPR or preimmune sera. Monolayers of Vero cells were labeled for 6 h with 50 μCi of [^{35}S]methionine per ml. NP-40–DOC lysates from the cells were mixed with either antibodies from preimmune rabbits or rabbits injected with the 46- and 275-kDa MPR from bovine testes. Immunoprecipitated proteins were subjected to electrophoresis on 7.5% polyacrylamide gels, and the gels were exposed to X-ray film. Sizes are indicated in kilodaltons. (D) Inhibition of HSV plaques by purified anti-MPR or anti-gD antibodies. Vero cells were incubated for 30 min at 32°C with various concentrations of IgG purified from pooled rabbit preimmune serum (●), pooled anti-gD sera (▲), or pooled anti-MPR sera (□) diluted in α-MEM containing 1% FBS. HSV-2 was added for a further 90 min at 37°C, then the virus and media were removed, and the cells were treated with citrate buffer (pH 3.0) for 1 min. The cells were washed and incubated for 36 h at 37°C and then stained with crystal violet, and plaques were counted.

manner. In four experiments, HSV-2 plaques were inhibited in number by 45 to 75% when virus was treated with 6 μM MPR. Preparations of the 225-kDa MPR which had been heat denatured did not affect the number of HSV-2 plaques, and there was no evidence that the purified 225-kDa MPR produced any toxicity to cells which could be cultured in the presence of 6 μM MPR for 5 days without changes in morphology or growth (results not shown). In other experiments, similar inhibition of HSV-1 plaques was observed when virus was mixed with soluble 225-kDa MPR (not shown).

Rabbit anti-MPR antibodies inhibit production of HSV-2 plaques. To characterize the role of MPRs in HSV entry further, polyclonal anti-MPR antibodies were produced in rabbits. The 275- and 46-kDa MPRs were purified from bovine testes by PM-PO_4 affinity chromatography as previously described (16, 37). Again, a high degree of purity was obtained in a single step. SDS-polyacrylamide gel electrophoresis of these preparation, followed by Coomassie blue staining, demonstrated that the 275- and 46-kDa MPRs were by far the predominant proteins in these preparations (Fig. 2A). There were, however, faint traces of contaminating proteins of approximately 70 kDa in these preparations. Rabbits were injected with purified MPRs or, in parallel, with soluble gD-2t (30). When the purified MPRs were subjected to electrophoresis, the anti-MPR antibodies reacted with the 275- and 46-kDa MPRs on Western blots (Fig. 2B). In addition, when Vero cells were radiolabeled with [^{35}S]methionine and [^{35}S]cysteine, the anti-MPR serum precipitated both the 275- and 46-kDa MPRs as well as a protein of approximately 85 kDa (Fig. 2C). This protein was also detected with an anti-MPR serum from another laboratory (49a). This 85-kDa protein may represent a proteolytic product of the 275-kDa MPR, a cross-reactive cellular protein, or a protein contaminant of the original MPR preparation. The anti-MPR sera were of relatively low titer compared with anti-gD sera. For example, in dot blot assays in which gD-2t or purified MPRs were bound to nitrocellulose, anti-MPR antibodies could be detected at a dilution of 1:500, whereas anti-gD-2t antibodies could be detected at a dilution of 1:10,000. We expect that these differences in antibody titers are related to difficulties in producing high-titer sera to the relatively conserved MPRs. In contrast, gD is highly antigenic. IgG fractions were purified from the anti-MPR, preimmune, and anti-gD sera.

The effects of anti-MPR antibodies were tested by incubating the IgG with Vero cell monolayers for 30 min at 32°C and then adding HSV-2 for 90 min at 37°C. Unabsorbed virus and unbound sera were removed, and then the cells were washed with citrate buffer (pH 3.0) to inactivate HSV-2 which had adsorbed but not entered cells. The cell monolayers were in-

cubated for an additional 36 h, the cells were stained, and plaques were counted. Anti-MPR IgG inhibited production of HSV-2 plaques by greater than 55% at the highest concentrations used (400 μg/ml) (Fig. 2D) and was not obviously toxic to cells. Similar results were obtained in other experiments with purified IgG derived from an anti-MPR serum described previously (18), and there was no effect of the anti-MPR antibodies if the antibodies were added after virus entry into cells (not shown). However, we cannot discount the possibility that the anti-MPR antiserum blocks HSV entry by binding to a cross-reactive protein, such as the 85-kDa protein that we observed in Fig. 2C. Anti-gD IgG was more effective in blocking plaque formation, with 50% inhibition observed at the lowest concentrations tested (25 μg/ml) and no plaques detected at 200 μg/ml. There was little or no effect on the number of HSV-2 plaques when Vero cells were incubated with IgG derived from preimmune sera at 400 μg/ml (Fig. 2D). Similar inhibition of HSV-2 plaques was observed on monolayers of bovine MDBK cells treated with anti-MPR sera (not shown).

Inhibition of HSV plaques by PM-PO_4-BSA. Binding of lysosomal enzymes to cell surface MPRs can be at least partially inhibited by M-6-P, albeit the affinity of both MPRs for monomeric M-6-P is relatively low (8×10^{-6} M). Diphosphorylated oligosaccharides bind to the 275- and the 46-kDa MPRs with higher affinities (2×10^{-9} and 2×10^{-7} M, respectively) suggesting that MPRs bind M-6-P residues at more than a single site (17, 59). We observed no inhibition of HSV plaque formation when cells and virus were incubated with 10 μM M-6-P or with 1 μM IGF-II (not shown). To evaluate a ligand which is bulky and binds to both MPRs with higher affinity than does M-6-P, we purified PM-PO_4 from yeast phosphomannan and conjugated this material to BSA, producing PM-PO_4-BSA, which binds specifically and with high affinity to cell surface MPRs (4, 57). The extent of BSA modification with PM-PO_4 was approximately 30 to 35 PM-PO_4 molecules per protein molecule, as judged from the substantial decrease in electrophoretic mobility observed on SDS-polyacrylamide gels (85 to 90 kDa versus 68 kDa for unmodified BSA) (Fig. 3A, inset).

We then tested the ability of PM-PO_4-BSA to block HSV entry into cells. Vero cells were incubated with various concentrations of PM-PO_4-BSA for 40 min, and then HSV-1 or a control virus, VSV, was added (~100 PFU/2-cm^2 well) for a further 60 min in the presence of PM-PO_4-BSA. Unbound virus was removed, and the cells were washed and incubated for 36 h (HSV-1) or 18 h (VSV) in the presence of the appropriate concentration of PM-PO_4-BSA. In the experiment shown, there was a reduction in the number of HSV-1 plaques produced by over 80% when cells were treated with 4.5 μM (corresponding to ~400 μg/ml) PM-PO_4-BSA throughout the experiment and of 75% when the cells were treated with 2.3 μM PM-PO_4-BSA (Fig. 3A). Cells treated with similar concentrations of BSA alone showed no inhibition of HSV plaque production (not shown). When PM-PO_4-BSA was present only during the first 100 min (Fig. 3A), there was little or no inhibition of HSV plaques, suggesting that virus adsorption onto the cells was not inhibited by this treatment and virus particles could enter cells once PM-PO_4-BSA was removed. Similarly, the number of HSV plaques was not altered when PM-PO_4-BSA was added after virus entry into cells (Fig. 3A), suggesting that PM-PO_4-BSA does not block postentry stages of HSV replication in cells. In these experiments, replicate wells were not analyzed because materials were limited; however, three experiments using different preparations produced inhibition of HSV-1 ranging from 61 to 82%. Treatment of Vero cells with PM-PO_4-BSA not only inhibited the number of HSV plaques produced on the cells but also substantially reduced

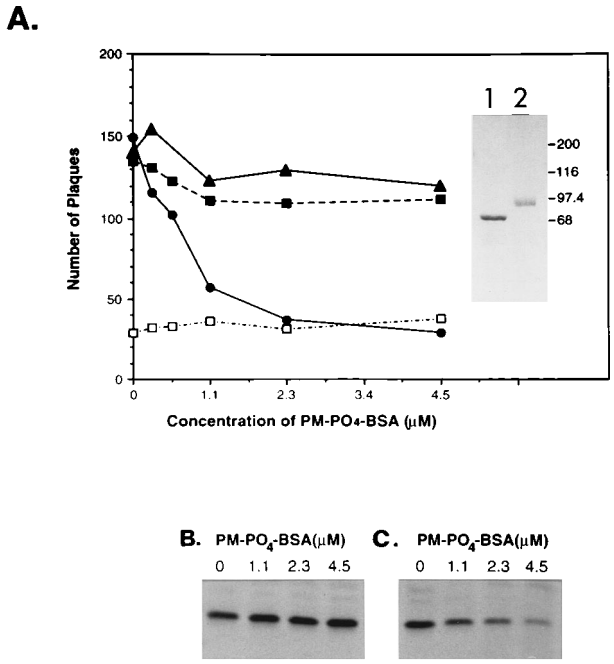

FIG. 3. Inhibition of HSV-1 plaque production and virus entry by the MPR ligand PM-PO_4-BSA. (A) Vero cell monolayers growing in 12-well dishes were treated with 0, 0.3, 0.6, 1.1, 2.3, or 4.5 μM PM-PO_4-BSA for 40 min at 37°C. Cells were infected with either HSV-1 (solid symbols) or VSV (open symbols) for 60 min at 37°C, then unbound virus and PM-PO_4-BSA were removed, and the cells were washed. Cells infected with HSV-1 were incubated for 36 h with medium either containing (●) or lacking (■) the appropriate concentration of PM-PO_4-BSA. Other monolayers were treated with various concentrations of PM-PO_4-BSA for 36 h only after the virus inoculum was removed (▲). The cells were then stained with crystal violet, and HSV plaques were counted. Vero cells infected with VSV were overlaid with medium containing 1% agarose and the appropriate concentration of PM-PO_4-BSA. VSV plaques were allowed to develop over 18 h and visualized by addition of a solution of 1% neutral red. The inset shows a polyacrylamide gel in which BSA (lane 1) and PM-PO_4-BSA (lane 2) were subjected to electrophoresis and then stained with Coomassie blue; sizes are indicated in kilodaltons. (B and C) Vero cells were treated with various concentrations of PM-PO_4-BSA for 30 min, and then HSV-1 (10 PFU per cell) was added for an additional 90 min at 37°C. The virus and PM-PO_4-BSA were removed, and the cells were washed and incubated with medium lacking PM-PO_4-BSA (B) or incubated with medium containing the appropriate concentration of PM-PO_4-BSA (C). After 3 h, the cells were radiolabeled with [^{35}S]methionine for 2 h, and HSV thymidine kinase was immunoprecipitated from cell extracts.

the sizes of HSV-1(F) plaques produced in the presence of 1.1 or 2.3 μM PM-PO_4-BSA (Fig. 4). In contrast to the inhibition of HSV, PM-PO_4-BSA did not inhibit production of plaques by VSV (Fig. 3A). PM-PO_4-BSA was not toxic to cells and produced no detectable alteration in the growth or morphology of uninfected Vero cells over several days (results not shown).

There was also evidence that PM-PO_4-BSA inhibited the initial entry of HSV-1 into cells, rather than some other aspect of virus replication required to form plaques. Cells were treated with various concentrations of PM-PO_4-BSA, then infected with HSV at 10 PFU per cell, and subsequently radiolabeled with [^{35}S]methionine. The HSV-1 early protein thymidine kinase was immunoprecipitated from cell extracts as a measure of virus entry into cells (Fig. 3B and C). In this experiment, when PM-PO_4-BSA was kept continuously present, there was inhibition of early protein synthesis (Fig. 3C), but when the PM-PO_4-BSA was removed after virus adsorption (Fig. 3B), some fraction of the HSV-1 entered the cells once the inhibitor was removed and there was little or no inhibition of thymidine kinase expression.

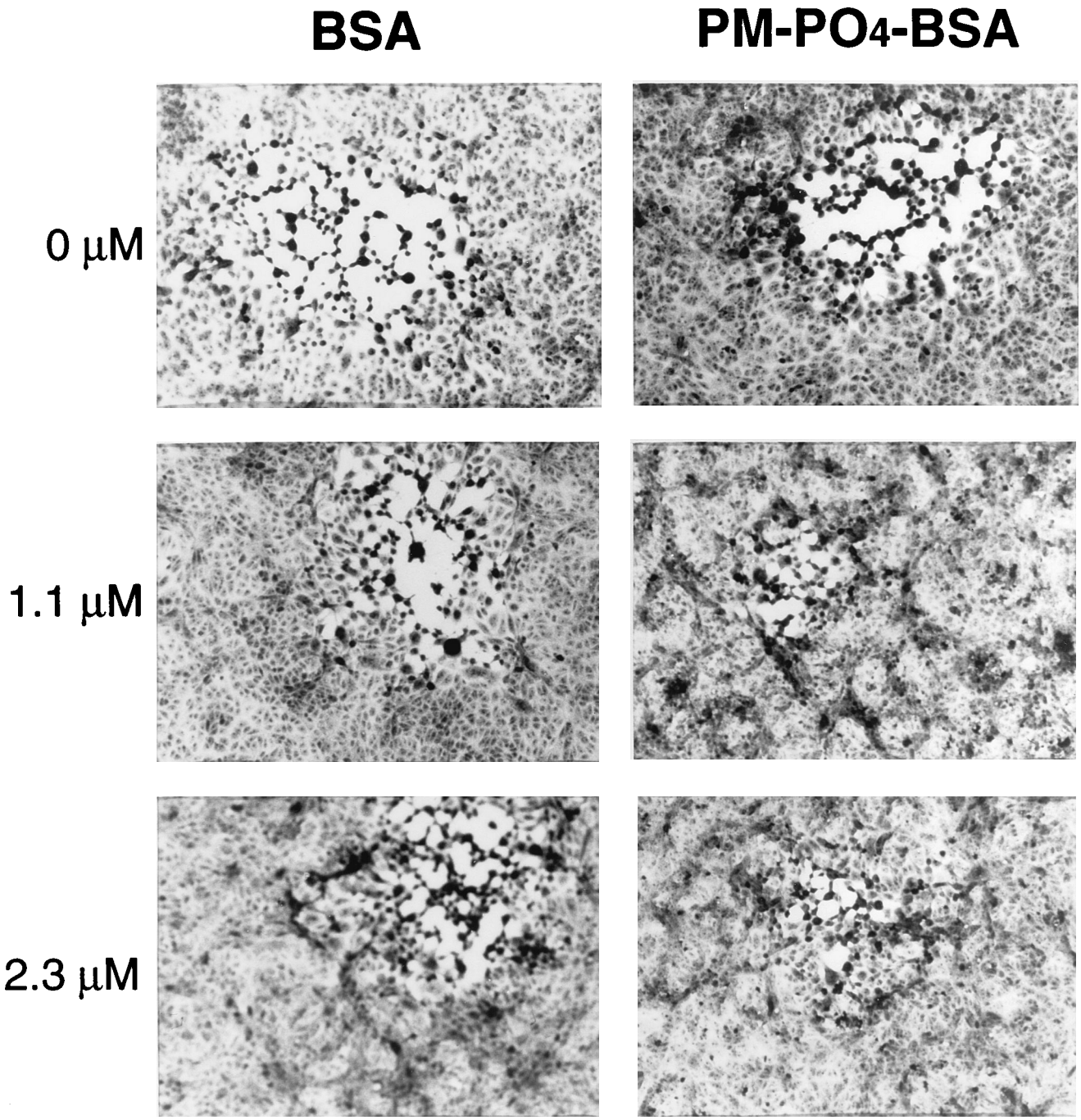

FIG. 4. PM-PO_4-BSA reduces the size of HSV-1 plaques. Vero cell monolayers were incubated with 0, 1.1, or 2.3 μM PM-PO_4-BSA or the same concentrations of BSA for 90 min at 37°C and then the cells were infected with HSV-1(F) for 2 h in the presence of inhibitors. The cells were washed and incubated for a further 48 h in medium containing 0.2% HGG and the appropriate concentration of PM-PO_4-BSA or BSA. Representative plaques were photographed after staining with crystal violet.

The experiments in Fig. 3 and 4 involved HSV-1, whereas the results in Fig. 1 and 2 involved HSV-2. We have not detected differences in the entry of HSV-1 and HSV-2 into cells: UV-inactivated HSV-2 blocks entry of HSV-1 and vice versa (31), HSV-1 gD blocks entry of both HSV-2 and HSV-1, and HSV-2 gD inhibits both HSV-1 and HSV-2 (30), and both proteins bind to MPRs (5). Therefore, we assume that HSV-1 and HSV-2 are functionally interchangeable in these particular experiments, and for technical reasons, e.g., the anti-MPR serum was compared with serum directed to HSV-2 gD, we primarily used HSV-2 in early experiments and HSV-1 in the later experiments. Given limited quantities of material, it was frequently difficult to compare HSV-1 and HSV-2 extensively, and in actuality, it may be more important to compare the effects of these inhibitors on different cell types.

Infection of MPR-deficient mouse fibroblasts by HSV. To further assess the role of MPRs in HSV entry into cells, we studied mouse fibroblasts unable to express one or both of the

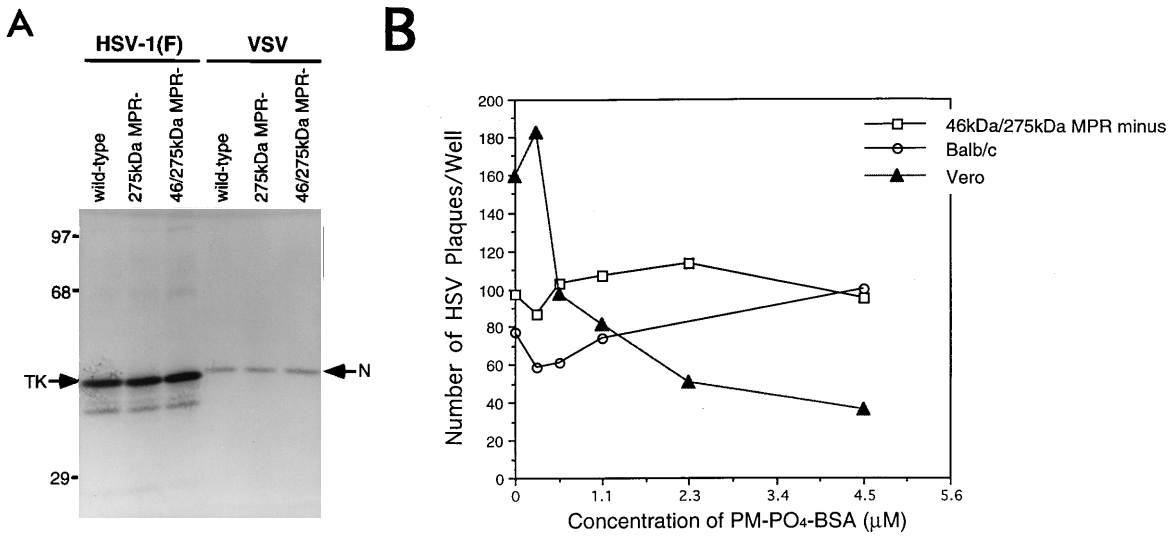

FIG. 5. HSV-1 replication in MPR-deficient mouse fibroblasts. (A) Monolayers of normal mouse fibroblasts, fibroblasts from 275-kDa MPR-deficient mice (275-kDa MPR-), or fibroblasts from mice lacking both the 46- and 275-kDa MPRs were infected with HSV-1(F) or VSV at 1 PFU per cell and incubated at 37°C for 2 h. The cells were labeled with [^{35}S]cysteine and [^{35}S]methionine (100 μCi of each per ml) for 3 h. HSV thymidine kinase (TK) was immunoprecipitated from HSV-1-infected cell extracts, and the VSV N protein was immunoprecipitated from extracts of VSV-infected cells. Proteins were resolved on 10% polyacrylamide gels. Sizes are indicated in kilodaltons. (B) Monolayers of normal (○) or 46/275-kDa MPR-deficient fibroblasts (□) or Vero cells (▲) growing in 12-well dishes were treated with 0 to 4.5 μM PM-PO_4-BSA for 40 min at 37°C, and then HSV-1(F) (~100 PFU per well) was added for a further 60 min at 37°C. Unbound virus and PM-PO_4-BSA were removed, the cells were washed twice with medium and incubated for 48 h in medium containing 0.2% HGG and the appropriate concentration of PM-PO_4-BSA, the cell monolayers were stained with crystal violet, and plaques were counted.

MPRs (39). These fibroblasts were derived from embryos of mice lacking the 275-kDa MPR or from mice lacking both the 275- and 46-kDa MPRs. There is evidence that lysosomal enzymes are missorted in these cells and secreted rather than being targeted to lysosomes. We infected fibroblasts derived from wild-type (BALB/c) mice, mice lacking the 275-kDa MPR, or mice lacking both the 46- and 275-kDa MPRs with HSV-1(F) or with VSV; 2 h later, the cells were labeled with [^{35}S]cysteine and [^{35}S]methionine. To quantitate virus entry into the cells, the HSV early protein thymidine kinase or the VSV N protein were immunoprecipitated from cell extracts. Similar amounts of thymidine kinase were produced in HSV-infected wild-type, 275-kDa MPR-deficient, and 46/275-kDa MPR-deficient fibroblasts (Fig. 5A). The levels of VSV N protein produced in all three cell lines were also similar. In other experiments, there was no significant difference in the number of HSV plaques produced on wild-type and MPR-deficient fibroblasts. Because the plaques formed on these embryonic mouse fibroblasts were not large, it was difficult to ascertain whether the plaques formed on MPR-deficient fibroblasts were smaller than those formed on the normal mouse fibroblasts.

The ability of PM-PO_4-BSA to inhibit HSV entry was tested on both normal and MPR-deficient mouse fibroblasts. These experiments provided a suitable control for toxicity of PM-PO_4-BSA on virus and cells and also tested whether MPRs play a role in HSV entry into these mouse fibroblasts. PM-PO_4-BSA did not inhibit HSV plaque formation on either normal mouse fibroblasts or 46/275-kDa MPR-deficient fibroblasts, yet there was inhibition on Vero cells in the same experiment (Fig. 5B). In other experiments, PM-PO_4-BSA had no effect on HSV entry into normal mouse fibroblasts or 46/275-kDa MPR-deficient fibroblasts as assessed by expression of thymidine kinase in the cells (data not shown). These results further confirm that HSV does not rely on MPRs to enter these embryonic mouse fibroblasts. These observations also suggest that there are differences in entry of HSV into monkey Vero cells and these mouse fibroblasts. In the experiment shown, the number of HSV plaques produced on both types of mouse fibroblasts was lower than produced on Vero cells, suggesting that infection of the mouse cells may be less efficient. In addition, since there was no measurable effect of PM-PO_4-BSA on either the mouse cells or virus, the results further support our belief that this inhibitor is not generally toxic to cultured cells or to HSV replication.

In the absence of gD phosphorylation, HSV produces small plaques on fibroblasts monolayers. Fibroblasts derived from patients with pseudo-Hurler polydystrophy possess very low levels of *N*-acetylglucosamine-1-phosphotransferase activity, which is required for phosphorylation of mannose residues on lysosomal enzymes (35, 47). Modification of lysosomal enzymes with M-6-P in pseudo-Hurler fibroblasts is reduced to 2% of the level found in normal fibroblasts (60a). There is little or no information on the status of MPRs in pseudo-Hurler fibroblasts, although one would not expect alterations in these proteins. Presumably, HSV gD, which has three N-linked glycosylation sites, would be inefficiently modified with M-6-P in pseudo-Hurler fibroblasts. To determine the effects of incomplete phosphorylation of gD, fibroblasts derived from a pseudo-Hurler patient (GM3391) or fibroblasts from a healthy parent of this patient (GM0080) were infected with HSV-1, and virus plaques were allowed to form over a period of 56 h. Wild-type HSV-1(F) produced similar numbers of plaques on both pseudo-Hurler fibroblasts and normal human fibroblasts. However, smaller plaques were produced by HSV-1(F) on pseudo-Hurler fibroblasts (10 to 20 cells infected) compared with plaques produced on normal human fibroblasts (>100 infected cells) (Fig. 6A and C); similar results were obtained with HSV-2 (not shown). As a control in these experiments, we used HSV-1 QAA, a mutant derived from HSV-1(F) in which gD coding sequences were altered so that none of the three N-linked oligosaccharides are added (54). Since there are no N-linked oligosaccharides and O-linked oligosaccharides cannot be modified with M-6-P (34), it is very likely that the QAA

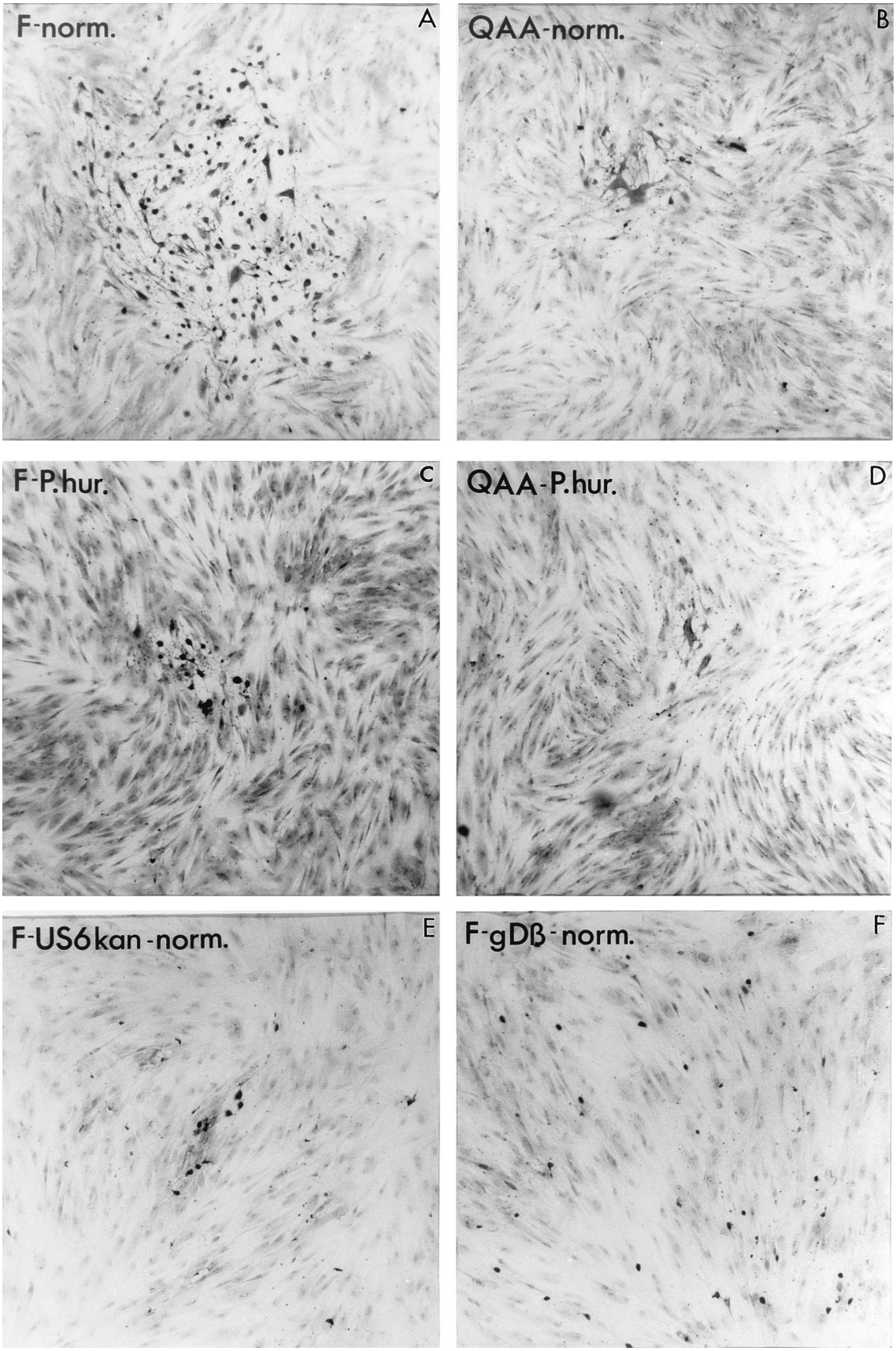

FIG. 6. HSV plaques on normal and pseudo-Hurler fibroblasts. Monolayers of normal (norm) or pseudo-Hurler (P.hur.) fibroblasts were infected with wild-type HSV-1 strain F or HSV-1 mutant QAA, which expresses a form of gD lacking N-linked oligosaccharides, F-US6kan, which does not express gD, or F-gDβ, which does not express gD and gI. F-US6kan and F-gDβ virus preparations were derived from VD60 cells. Cells were stained with crystal violet after 56 h, and representative plaques were photographed.

gD molecule is not modified with M-6-P in both pseudo-Hurler and normal fibroblasts. The QAA mutant produced small plaques (10 to 20 infected cells) on both normal and pseudo-Hurler fibroblasts (Fig. 6B and D). These observations supported the notion that the small-plaque phenotype of wild-type HSV-1 on pseudo-Hurler cells was related to lack of phosphorylation of gD rather than to other effects.

An HSV-1 mutant unable to express gD, F-US6kan, also produced very small plaques on normal human fibroblasts; three to five cells were infected after 56 h (Fig. 6E). A second HSV-1 gD mutant, F-gDβ, which is also unable to express gI, was unable to spread beyond a single cell (Fig. 6F). Note that the F-US6kan and F-gDβ virus stocks used to infect these fibroblasts were derived from complementing VD60 cells which supply gD in *trans*, and thus viruses initiating the infection possessed gD. These results demonstrated that HSV-1 can spread, albeit inefficiently, from cell to cell without gD and produce microscopic plaques. However, efficient spread of HSV requires gD molecules, and defects in glycosylation or the addition of M-6-P to gD (and perhaps other HSV glycoproteins) markedly reduced cell-to-cell spread. The observation that F-gDβ (which lacks gI as well as gD) was unable to form any type of plaque, i.e., to spread at all, is consistent with previous observations that the gE-gI hetero-oligomer contributes substantially to the ability of HSV to spread from cell to cell in monolayers of human fibroblasts (15).

It was possible that the small plaques produced by HSV-1 on pseudo-Hurler fibroblasts were related to defects in virus replication in the cells. To address this point, normal and pseudo-Hurler fibroblasts were infected with wild-type HSV-1(F) at 10 PFU per cell; at various times after infection, the cells and growth media were harvested and infectious HSV-1 was quantitated by using plaque assays on Vero cells. The kinetics of production of infectious HSV-1 were similar on both normal and pseudo-Hurler fibroblasts, and the final amounts of virus produced were also similar (Fig. 7). These data suggested that the small-plaque phenotype of HSV-1 on pseudo-Hurler fibroblasts was due to a defect in cell-to-cell spread of virus and not due to defects in virus replication in the cells. In addition, the results show that HSV-1 derived from pseudo-Hurler fibroblasts, in which gD is inefficiently modified with M-6-P, produces normal numbers of plaques on Vero cell monolayers.

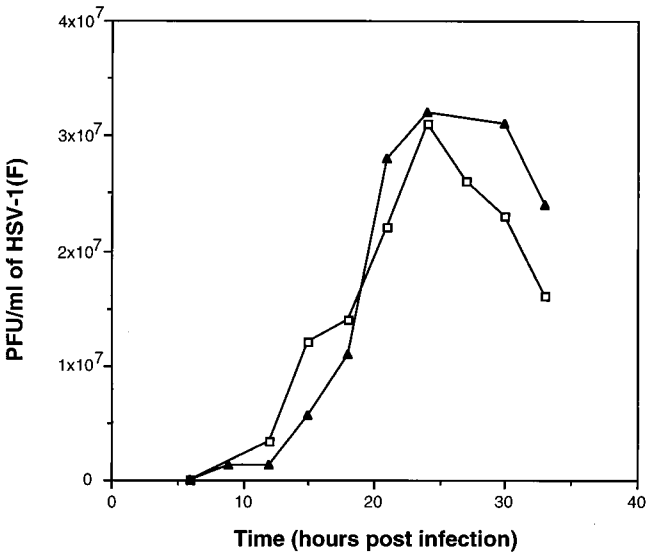

FIG. 7. Similar numbers of infectious HSV particles are produced on normal and pseudo-Hurler fibroblasts. Monolayers of normal (□) and pseudo-Hurler (▲) fibroblasts were infected with HSV-1(F) at 10 PFU per cell; at various times after infection, the cells were scraped into the medium and sonicated. Infectious HSV was quantitated by plaque titration on monolayers of Vero cells.

DISCUSSION

There is considerable evidence that HSV gD is required for virus entry into cells, promoting virus interactions with a relatively restricted set of cell surface receptors at a stage subsequent to adsorption onto cell surface GAGs. To identify the cellular receptors with which gD interacts, we developed a ligand blot assay, and using this assay coupled with gD affinity chromatography, we purified a large cellular membrane protein which was identified as the 275-kDa MPR (5). Purified MPRs could bind to both soluble and viral gD molecules, though the binding to viral gD was low and only a small fraction of viral gD was modified with M-6-P, whereas virtually every molecule of soluble gD was phosphorylated. It is possible that gD molecules in the virion envelope are more extensively modified with M-6-P than gD extracted from virus-infected cells; however, efforts to analyze gD from virions have, to date, been unsuccessful. Nevertheless, there are thousands of gD molecules per virion, and thus a relatively low level of phosphorylation may have important biological implications for HSV. It is also possible that gD is inefficiently modified with M-6-P in order to promote escape of newly produced virions so that virus particles are not retained in endosomal or lysosomal membranes or on cell surface MPRs. We note that a small fraction of varicella-zoster virus (VZV) glycoproteins are also modified with M-6-P, and it has been hypothesized that this causes VZV to remain primarily cell associated (22). Like VZV, HSV remains primarily cell associated. In analyzing the phosphorylation of gD, we observed that soluble gD-2t could be modified in vitro by *N*-acetylglucosamine-1-phosphotransferase, the enzyme responsible for the addition of M-6-P residues, and the relative catalytic efficiency of this reaction was greater than that for cathepsin D, an authentic lysosomal enzyme (5). These results suggest that modification of gD, and perhaps other herpesvirus glycoproteins, with M-6-P is not fortuitous and has important implications for the biology of HSV and other herpesviruses.

To investigate the role of MPRs in HSV entry into monkey Vero cells, we investigated the effects of a synthetic ligand of MPR, anti-MPR antibodies, and a soluble form of the 275-kDa MPR. Incubation of HSV with soluble MPR inhibited plaque production by as much as 75% in some experiments, though the inhibition was less (45%) in other experiments. On the assumption that only a small fraction of virion gD molecules are modified by M-6-P residues (5), it is likely that those gD molecules that are modified are monophosphorylated rather than diphosphorylated. Since proteins substituted with only a single M-6-P residue bind to surface MPRs with lower affinities than proteins with multiple M-6-P substitutions, we would expect that soluble MPR binds to viral gD molecules with an affinity of only ~8 μM (17, 59, 60). Given this consideration, it may not be surprising that there was only partial inhibition of HSV entry and that this required 6 μM soluble MPR.

Polyclonal antisera raised against both the 275- and 46-kDa MPRs inhibited HSV plaque production by up to 55%. In interpreting the results with these sera, a number of points must be considered. It is well known that only a small fraction of MPRs (5 to 10%) are present on the cell surface at any one time and that the receptors recycle rapidly (23, 45, 56). Thus, MPR ligands or anti-MPR antibodies may be rapidly removed from the cell surface by recycling. Furthermore, MPRs are

highly conserved among mammalian species, and the titers of our anti-MPR sera were about 20-fold lower than titers of anti-gD sera prepared in parallel and which more effectively inhibited HSV plaque production. In comparing these two sets of antisera, it should be made clear that the anti-gD antibodies can neutralize HSV or block entry by binding directly to virus particles, which is unlikely to be the case for anti-MPR antibodies, which must bind to surface MPRs constantly recycling from within cells. Furthermore, it has been difficult to inhibit binding and uptake of M-6-P-modified proteins by more than 50% with similar anti-MPR antibodies (57). Recent observations with adenoviruses highlight the difficulty of inhibiting virus entry by using antireceptor antibodies. Anti-integrin antibodies used at concentrations similar to those used in this study (100 to 500 μg/ml) caused only partial inhibition of adenovirus entry into cells (43, 62), perhaps because adenoviruses can enter by using other receptors, although other explanations are also possible because cells lacking receptors are much more resistant to adenovirus.

Inhibition of HSV plaque production and virus entry into cells was also observed when cells were treated with PM-PO_4-BSA, which acts as a high-affinity, relatively bulky ligand for surface MPRs. As with soluble MPR, concentrations of 1.1 to 4.5 μM PM-PO_4-BSA were required to inhibit HSV entry and plaque formation by 61 to 82%. Again, there was still a substantial fraction of HSV which could enter cells treated with even the highest concentrations of PM-PO_4-BSA tested. These inhibitory concentrations are similar to those of soluble gD-2t (2 to 4 μM) required to inhibit HSV entry into cells and plaque production (30). Given that the affinity of soluble gD-2t for cell surface receptors is only moderate ($K_D \approx 0.25$ μM [30]), it is perhaps not surprising that 10- to 20-fold-higher concentrations of gD-2t, or PM-PO_4-BSA, might be required to inhibit interactions between virus and MPRs, interactions that are likely to be highly multimeric. Similar concentrations of soluble gB-2t, which is not modified with M-6-P (5), did not alter plaques (30).

To inhibit HSV entry into cells, it was necessary to keep PM-PO_4-BSA present continuously, as was also the case with soluble MPR. We found that there was little or no effect on plaque formation if PM-PO_4-BSA was present during the period in which cells and virus were incubated together but was removed when the virus inoculum was removed. We interpret these results to indicate that virus particles linger on surface GAGs and enter cells when MPRs are no longer blocked. Similarly, soluble gD must be kept continuously present in order to inhibit virus entry and soluble gD had no effect on HSV adsorption onto the cell surface (30), suggesting, again, that HSV stalled on surface GAGs can enter cells once soluble gD is removed. In addition, the continuous presence of PM-PO_4-BSA was not toxic to cells and did not inhibit virus replication at a postentry stage because there was no inhibition by PM-PO_4-BSA which was continuously present 60 min after addition of HSV. It might be suggested that PM-PO_4-BSA, which possesses a negative charge, can inhibit HSV entry analogously to heparin, by inhibiting the ability of virus to adsorb onto cell surface heparan sulfate GAGs (55, 64). However, there are important differences in how PM-PO_4-BSA and heparin act. Heparin will effectively inhibit HSV adsorption onto the cell surface, if present only during the period when virus and cells are incubated together (64), and yet PM-PO_4-BSA must be kept continuously present in order to inhibit HSV. In addition, there was no effect of PM-PO_4-BSA on mouse fibroblasts, cells which presumably contain heparan GAGs since heparin blocks HSV infection of mouse cells (24). Therefore, our results with three inhibitors, PM-PO_4-BSA, soluble MPR, and anti-MPR antibodies, support the hypothesis that HSV utilizes MPRs to enter cells and that this occurs at a postadsorption step. However, since in none of these experiments could we completely inhibit HSV entry or plaques, it is probable that other receptors or pathways must also function to mediate entry into human and monkey cells.

We also found that mouse fibroblasts lacking both MPRs were as efficiently infected by HSV as fibroblasts which express MPRs, and virus produced normal numbers of plaques on the MPR-negative cells. At face value, these results appear to contradict the results with soluble MPR, MPR ligands, and antibodies, arguing that HSV does not require MPRs to enter cells. However, it appears that there are MPR-independent entry pathways in monkey and human cells, and therefore these entry mechanisms may be predominant in these embryonic mouse fibroblasts. Indeed, M-6-P-independent uptake of phosphorylated lysosomal enzymes is quite important compared with M-6-P dependent uptake in these mouse embryonic fibroblasts (27a). Consistent with the idea that HSV enters these mouse cells primarily by MPR-independent pathways, little or no inhibition of HSV plaque production by PM-PO_4-BSA was observed on these fibroblasts. In addition, HSV plaque production on these mouse cells was frequently less efficient than with Vero cells. However, it is also clear that gD is important in entry into these mouse cells because gD-negative viruses could not enter the cells and recombinant gD-2t reduced the number of HSV plaques produced on these cells (4a). These results are consistent with the notion that gD has other functions, e.g., in membrane fusion (21), or binds to other receptors.

The phenotype of the QAA mutant, which lacks N-linked oligosaccharides and thus M-6-P residues on gD, is very different from that of HSV-1 gD-negative mutants. QAA produces normal numbers of plaques and penetrates into cultured monkey cells with similar kinetics to that of wild-type HSV-1 (54). Furthermore, HSV derived from pseudo-Hurler fibroblasts, which presumably lacks most or all M-6-P, can infect Vero cells and produce normal numbers of plaques. Thus, loss of phosphorylation of gD (and perhaps other HSV glycoproteins) causes effects different from those caused by ligand or antibody blocking of MPRs. Although we do not understand these results, they may suggest that MPR ligands and anti-MPR antibodies act not simply by binding to surface MPRs, blocking their availability to HSV. It is possible that the effects of inhibitors are indirect; for example, the inhibitors may sterically hinder other molecules that HSV uses to enter cells.

One way of explaining these results is to consider the observation that MPRs are primarily localized to cell surface coated pits so that they are highly restricted in terms of distribution and mobility (45). Anti-MPR antibodies and ligands added to the cell surface presumably bind to coated pits. If one assumes that HSV can use coated pits as part of its entry pathway, inhibition of HSV entry by MPR antibodies or MPR ligands may be explained by an indirect effect on coated pits. In this scenario, other HSV receptors may reside in coated pits and MPR ligands or anti-MPR antibodies may limit accessibility of these receptors. There is no published evidence suggesting that coated pits play a role in HSV entry into cells, and in contrast to this notion, there is evidence that HSV can enter cells by fusion of the virion envelope with the plasma membrane. Normally, viruses which use coated pits become endocytosed and enter the cytoplasm after fusion in low pH endosomes (26, 61). However, HSV does not appear to require low pH to fuse with cellular membranes because chloroquine does not block the virus (63). In addition, there is electron microscopic evidence for fusion of HSV particles with the plasma membrane (21),

although electron microscopy studies do not differentiate between infectious and noninfectious viruses. Nevertheless, since the entry pathway of HSV is so poorly characterized, it is reasonable to consider plasma membrane coated pits or pH-neutral endosomes as sites of HSV entry into the cytoplasm. Consistent with this hypothesis, we have obtained recent evidence that postadsorption stages of HSV entry can be inhibited by >90% when clathrin-coated cages are briefly disrupted (4b), though these treatments may have other effects. We also note that virus particles were found to accumulate in endosomes after incubation with cells constitutively expressing gD (7), supporting the view that HSV can become incorporated into coated pits and endocytosed. As an alternative explanation, MPR-specific antibodies or ligands may inhibit intracellular stages of the virus entry pathway after these inhibitors enter cells by endocytosis.

It is also possible that gD binds to MPRs in an M-6-P-independent manner. Endoglycosidase F-treated gD, in which M-6-P would be removed, does not bind efficiently to MPRs (5), though there is some evidence that this gD may be partially misfolded or less stable (53). Endoglycosidase F-treated gD can block HSV plaque production when incubated with cells and virus (4b). However, further efforts to measure M-6-P-independent binding of gD to MPRs are under way. These results suggest that even if HSV uses MPRs to enter cells, gD performs another important function during virus entry.

The results from experiments involving human pseudo-Hurler fibroblasts, which underphosphorylate cellular and viral glycoproteins, suggest that modification of gD (and perhaps of other viral glycoproteins) with M-6-P is important for the direct cell-to-cell spread of HSV between human fibroblasts. There is growing evidence that cell-to-cell spread of HSV has properties that are distinct from entry of exogenous virus particles. For example, HSV mutants lacking glycoproteins E or I can efficiently enter human fibroblasts as extracellular virus particles, but these mutants cannot efficiently spread from cell-to-cell (1a, 15). Virus particles lacking gD (F-US6kan) form very small plaques on human fibroblasts, suggesting that gD is required for cell-to-cell spread of virus, although the process occurs inefficiently in the absence of gD. Since there were no defects in virus replication in the pseudo-Hurler cells and normal numbers of infectious virus were produced in these cells, it appears that modification of glycoproteins with M-6-P is important for some aspect of HSV cell-to-cell transmission. Further confirmation that this defect involved M-6-P modification of gD rather than some other defect in the cells came from the observation that the HSV-1 QAA mutant, which lacks N-linked oligosaccharides and M-6-P, also formed small plaques on both wild-type and pseudo-Hurler fibroblasts. Moreover, HSV plaques were smaller on Vero cells treated with PM-PO_4-BSA than on control Vero cells, suggesting that once virus is inside these monkey cells, cell-to-cell spread of the virus can be inhibited by blocking MPRs. Together, these results argue that the M-6-P and MPRs are important in cell-to-cell spread of HSV. It is possible that this defect in virus spread between cells involves missorting of virus particles or viral glycoproteins in infected cells. However, we have not detected changes in the distribution of virus particles in pseudo-Hurler fibroblasts versus particles in normal fibroblasts by electron microscopy (4b), and normal levels of infectious HSV are produced within the cells. An intriguing hypothesis suggests that MPRs interact with M-6-P-modified HSV particles in order to sort virions from the exocytic pathway into the endosomal pathway. Once present in the endosomal pathway, viruses may more readily access cell junctions in order to move directly from cell-to-cell. This hypothesis assumes that HSV can avoid or resist lysosomes by some mechanism. Studies of HSV interactions with lysosomes may be a good starting point for testing this hypothesis.

In summary, we have obtained evidence that gD and HSV interact with MPRs at the cell surface, at least as one pathway by which the virus enters cells. However, clearly HSV can enter in the absence of MPRs or when viral glycoproteins are not modified with M-6-P. The process by which HSV spreads from cell to cell may be mechanistically different from virus entry into cells, though these processes also share some properties, and we have presented evidence that M-6-P and perhaps MPRs play a role in cell-to-cell transmission of HSV.

ACKNOWLEDGMENTS

We especially thank M. Slodki (USDA, Peoria, Ill.) for the gift of *P. holstii* phosphomannan and for helpful discussions and advice. We are indebted to Stuart Kornfeld for helpful advice. We also thank Pat Spear for sharing unpublished results and for helpful discussions and comments on the manuscript, R. Eisenberg and G. Cohen for the QAA mutant, helpful advice, and patience, and G. Jourdian for antiserum. Tim Gregory, Michael Tigges, Craig Smibert, Lloyd Hutchinson, and Ian York all provided technical advice and moral support throughout the long course of this work. We acknowledge the excellent technical assistance of Steven Primorac, Philip Ng, and Cheryl Goldbeck.

This work was supported by a grant from the National Cancer Institute of Canada (NCIC) and the Medical Research Council of Canada. C.R.B. is the recipient of an NCIC studentship, and D.C.J. is an NCIC senior research scholar.

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