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Herpes Simplex Viruses Lacking Glycoprotein D Are Unable To Inhibit Virus Penetration: Quantitative Evidence for Virus-Specific Cell Surface Receptors

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Herpes simplex virus (HSV) glycoprotein D (gD) plays an essential role in the entry of virus into cells. HSV mutants unable to express gD were constructed. The mutants can be propagated on VD60 cells, which supply the viruses with gD; however, virus particles lacking gD were produced in mutant-infected Vero cells. Virus particles with or without gD adsorbed to a large number ($>4 \times 10^4$) of sites on the cell surface; however, virions lacking gD did not enter cells. Cells pretreated with UV-inactivated virions containing gD ($\approx 5 \times 10^3$ particles per cell) were resistant to infection with HSV type 1 (HSV-1) and HSV-2. In contrast, cells pretreated with UV-inactivated virions lacking gD could be infected with HSV-1 and HSV-2. If infectious HSV-1 was added prior to UV-inactivated virus particles containing gD, the infectious virus entered cells and replicated. Therefore, virus particles containing gD appear to block specific cell surface receptors which are very limited in number. Particles lacking gD are presumably unable to interact with these receptors, suggesting that gD is an essential receptor-binding polypeptide.

The earliest events in the replication of animal viruses include attachment to the plasma membrane and entry into the cytoplasm of host cells. Viral attachment components or receptor-binding proteins have been identified for a number of well-characterized viruses; however, in only a few cases have specific cell surface virus receptors been identified (3, 7, 11, 26, 30). Herpes simplex virus type 1 (HSV-1) specifies at least seven membrane glycoproteins, gB, gC, gD, gE, gG, gH, and gI (1, 4, 20, 25, 34, 36), which are the only viral polypeptides detected on the surfaces of virions and virus-infected cells. It seems likely that one or more of these envelope glycoproteins mediate attachment of virions to the cell surface; however, it is presently not clear which of these polypeptides participate in the adsorption process. Antibodies directed to gD and gC can inhibit virus adsorption to cells (12) and experiments involving liposomes composed of virion-derived glycoproteins have suggested a role for gB and gD in virus adsorption (22). However, viruses lacking gC, gB, or gD can adsorb to cells (5, 15, 18, 23), and thus none of these polypeptides is essential for virus adsorption. Virus receptors for HSV are apparently quite widespread because HSV can replicate in a variety of cell types derived from both primates and rodents. In addition, evidence has been presented that HSV-1 and HSV-2 bind to type-specific, saturable receptors (2, 39, 40).

Following attachment to the cell surface, viruses must pass across at least one cellular membrane so that the viral genome becomes available for virus replication. Enveloped viruses enter cells either by direct fusion with the plasma membrane at neutral pH or by fusion with endosomal membranes at acid pH (for reviews, see references 27, 37, 41). Entry of HSV appears to involve fusion of the virion envelope with the plasma membrane (13, 22, 32; M. Wittels and P. G. Spear, unpublished results). Antibodies to gD block virion envelope-plasma membrane fusion (13, 17, 29) and fusion of infected cells (31), suggesting that gD plays a role in virus penetration. In addition, gD when present in the plasma membrane of transfected cells can inhibit virus penetration but not virus adsorption to the cells (6). There is also evidence that gB and gH are essential for viruses entry or cell-cell spread (9, 14, 16, 24, 35) and that other gene products may regulate membrane fusion (8). Therefore, it appears likely that the binding and entry of HSV into cells is a multistep process involving more than a single virion envelope polypeptide and, perhaps, multiple cell surface receptors.

We previously described an HSV-1 mutant, F-gDβ, in which sequences encoding gD and a nonessential glycoprotein, gI, were replaced by *Escherichia coli* β-galactosidase sequences (23). This mutant produces virus particles lacking gD and gI which can bind to but not penetrate into cells. Here, we describe the construction of a second mutant, F-US6kan, which expresses gI but not gD. Both of these mutant viruses are propagated on a cell line, denoted VD60, which supplies the viruses with gD. We show that UV-inactivated virions containing gD were able to block the entry of HSV-1, whereas similar quantities of UV-inactivated virions lacking gD were unable to inhibit virus entry. These results suggest a model in which gD binds a limited set of cell surface receptors, an interaction which promotes virus penetration.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human R970-5 (R970) cells (33) were grown in α minimal essential medium (α-MEM) (GIBCO Laboratories, Burlington, Ontario, Canada) supplemented with 7% fetal calf serum (FCS). VD60 cells (23) were maintained in Eagle MEM lacking histidine supplemented with 1.0 mM histidinol (Sigma Chemical Co., St. Louis, Mo.) and 7% FCS. HSV-1 F and HSV G were obtained from P. G. Spear, Northwestern University, Chicago, Ill. The recombinants F-gDβ and F-US6kan were propagated on VD60 cells.

Antibodies. The HSV-1 gD-specific monoclonal antibody

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II-436 was a gift of P. G. Spear. Rabbit polyclonal serum specific for gD (10) was a gift of G. Cohen and R. Eisenberg, University of Pennsylvania, Philadelphia, Pa. Monoclonal antibody 15βB2 (21), which recognizes HSV-1 and HSV-2 gB, was a gift of S. Bacchetti, McMaster University, Hamilton, Ontario, Canada. Polyclonal rabbit serum, made in rabbits injected with crystalline HSV-1 thymidine kinase (TK) produced in *E. coli*, was a gift of W. Summers, Yale University, New Haven, Conn.

Construction of recombinant virus F-US6kan. Plasmid pUS6kan contains the *Bam*HI J fragment of HSV-1 where a kanamycin gene cassette is inserted at a *Hin*dIII site in the promoter of the gD gene (23). VD60 cells were cotransfected with pUS6kan and F-gDβ DNA as previously described (23). Recombinant viruses producing colorless plaques on VD60 cells which had been overlaid with medium containing 0.5% agarose and 300 μg of 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim Canada Ltd., Dorval, Quebec, Canada) per ml were isolated as previously described (23).

[^{35}S]methionine labeling of cells, immunoprecipitation, and gel electrophoresis. Vero or R970 cell monolayers were labeled with [^{35}S]methionine as previously described (19). Cell extracts were made at 6 to 8 h postinfection, and the extracts were sonicated, clarified by centrifugation, and mixed with mouse ascites fluid or rabbit serum and subsequently with protein A-Sepharose as described previously (19). Samples of precipitated proteins or cell extracts to which 2% sodium dodecyl sulfate (SDS), 2% β-mercaptoethanol, 10% glycerol, and bromophenol blue had been added were boiled for 5 min and then electrophoresed in 8.5 or 10% *N,N'*-diallytartardiamide cross-linked polyacrylamide gels (15); the samples were infused with 2,5-diphenyloxazole, dried, and exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.). Bands on X-ray film were scanned by using an LKB laser densitometer.

Purification of virions. [^{35}S]methionine-labeled or unlabeled virions were purified from Vero or VD60 cytoplasmic extracts by using dextran-T10 (Pharmacia, Inc., Piscataway, N.J.) gradients as previously described (23, 38) except that more cells (10^8) per gradient were used. A small fraction of the virion preparation was diluted in H_2O, and 4 μl of the diluted sample was dried on coated electron micrograph grids and then stained with phosphotungstinic acid. Virus particles were counted in photographs of the surface of the grids, and the number of particles was calculated by using photographs of a standard calibration grid. We found that virus particles were uniformly distributed on the surface of the grids.

UV inactivation and adsorption of cells with crude virus stocks or purified virions. VD60 or Vero cells infected with F-gDβ (5 PFU per cell) for 20 to 24 h were sonicated briefly, clarified by centrifugation at 1,200 × *g* for 10 min, and diluted to 1×10^9 to 3×10^9 PFU per ml with α-MEM plus 1% FCS. These preparations are referred to as crude virus stocks. Gradient-purified F-US6kan from VD60 or Vero cells was resuspended in α-MEM plus 1% FCS (1×10^9 to 3×10^9 PFU per ml) and sonicated briefly. Crude virus stocks or purified virions were exposed to a UV germicidal lamp producing principally 254-nm light at 4 J/m^2 per s for 15 min on ice with vigorous stirring. Subconfluent R970 cell monolayers in 24-well dishes were adsorbed with UV-inactivated virus stocks or gradient-purified virions diluted in α-MEM plus 1% FCS for 2 h on ice inside a CO_2 incubator. UV-inactivated virions were removed, and the cells were incubated with infectious HSV-1 strain F or HSV-2 strain G (10 PFU per cell) for 1 h at 4°C. The monolayers were washed, shifted to 37°C for 2 h, and then labeled with [^{35}S]methionine. After 6 to 7 h at 37°C, cell extracts were made, and these were stored at −70°C overnight and then used in immunoprecipitation reactions.

Binding of [^{35}S]methionine-labeled virions to cells. Confluent R970 cell monolayers in 96-well microdilution plates were exposed in duplicate to various concentrations of radiolabeled, gradient-purified virus diluted in α-MEM containing 10% FCS. After 3.5 h at 4°C, the cells were washed twice, and cell extracts were made by using Tris-saline (50 mM Tris hydrochloride [pH7.5], 100 mM NaCl) containing 0.5% SDS. Fractions containing unadsorbed virus, washes, and cell extracts were counted directly by using an aqueous scintillation fluid. Under these conditions, the amount of radiolabeled virus particles able to adsorb to plastic dishes was less than 10% of the virus which adsorbed to cell monolayers.

RESULTS

Construction of a recombinant HSV which expresses gI but not gD. The mutant virus F-gDβ lacks DNA sequences encoding gI and gD. Although gI is nonessential for replication in cultured cells (23, 25), studies of the role of gD in virus replication involving F-gDβ are complicated by the absence of gI in the virion envelope. For this reason, we constructed a recombinant virus able to express gI but not gD. By taking advantage of the fact that F-gDβ expresses β-galactosidase and thus produces blue plaques under agarose overlays containing X-Gal, plasmid sequences containing an interrupted copy of the gD gene and an intact gI gene were transferred into viral DNA. A plasmid, pUS6kan described previously (23), was constructed by inserting a kanamycin gene cassette between the promoter and structural sequences of the gD gene. This plasmid contains an intact gI gene. VD60 cells, which carry over 100 copies of the gD gene and can complement F-gDβ (23), were cotransfected with F-gDβ DNA and pUS6kan. The viral progeny from this transfection were screened for viruses which produced colorless plaques. A recombinant virus, F-US6kan, was isolated, and Southern blot analysis of plaque-purified F-US6kan (data not shown) indicated that the structure of the viral DNA in the region of the *Bam*HI J fragment was as depicted in Fig. 1. F-US6kan was unable to produce plaques on Vero cell monolayers and did not induce synthesis of gD in Vero cells (Fig. 2).

Previous studies (19, 20) have shown that gI interacts with gE to form a complex which has immunoglobulin G (IgG) Fc receptor activity. Rabbit IgG bound to protein A-Sepharose precipitates the gE/gI complex from extracts of virus-infected cells. Rabbit polyclonal antiserum (αgD) specific for gD precipitated the gE/gI Fc receptor complex as well as gD from extracts of F-US6kan-infected VD60 cells (Fig. 2). However, only the gE/gI complex was precipitated by αgD serum from extracts of F-US6kan-infected Vero cells. Thus, F-US6kan was able to express gI but did not express gD. We observed a smaller amount of the gE/gI complex precipitated by rabbit IgG in Vero cells relative to that observed in VD60 cells. This small difference may or may not be significant and may result from differences in the ability of antibody bound to antigen to interact with the Fc receptor. Alternatively, it is possible that HSV-infected VD60 cells express higher levels of gI because the cells have over 100 copies of the *Bam*HI J fragment, which includes all of the gI gene and part of the gE gene.

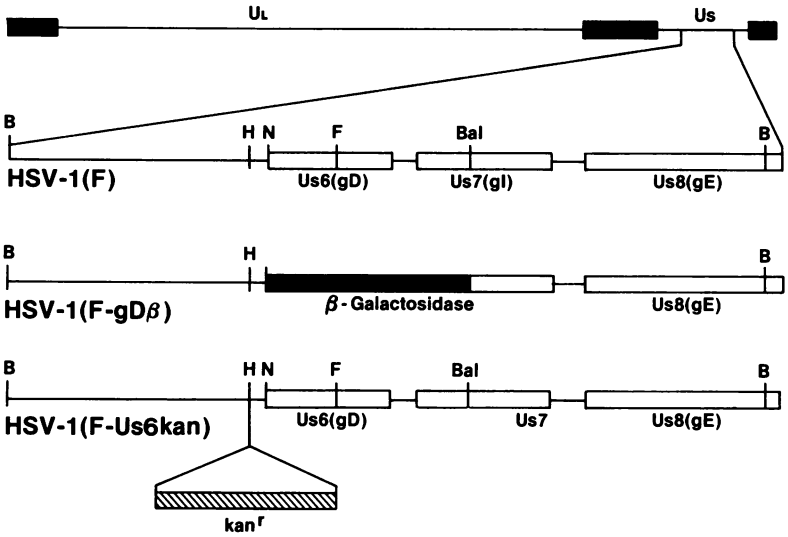

FIG. 1. Schematic representations of DNA sequence rearrangements in mutant strains F-gDβ and F-US6kan. The region of HSV-1 DNA, including the *Bam*HI J fragment, which contains the US6 (gD), US7 (gI), and US8 (gE) genes (28), is depicted for HSV-1 and recombinants F-gDβ and F-US6kan. In F-gDβ, β-galactosidase sequences replace gD structural sequences and part of the gI gene from an *Nco*I (N) site near the gD initiation codon to a *Bal*I (Bal) site in the gI gene. In F-US6kan, a kanamycin gene cassette is inserted at a *Hin*dIII (H) site in the promoter of the gD gene. B, *Bam*HI; F, *Fsp*I; U_L, unique long component of HSV-1; U_S, unique short component of HSV-1.

Inhibition of HSV infection by preadsorption of cells with virions containing or lacking gD. There is some evidence that cell surface receptors for HSV are saturable (2, 39, 40). To investigate the role of gD in the process of HSV attachment and entry into cells, we attempted to block HSV infection by preadsorbing cells with either virions containing gD or virions lacking gD. When the mutant viruses F-gDβ or F-US6kan are propagated on VD60 cells, gD is supplied by the cells and virions containing gD are produced (23). In Vero cells, F-gDβ and F-US6kan produced virions lacking gD. Both types of virus particles can adsorb to cells.

As a measure of the infectious process, the expression of TK was determined by immunoprecipitation of the protein by using a polyclonal antiserum produced in rabbits injected with crystalline TK. The level of TK expressed in HSV-1-infected cells was linearly related to the multiplicity of infection used, between 0.5 and 20 PFU per cell (Fig. 3). Therefore, TK expression serves as a direct measure of the efficiency with which UV-inactivated viruses block infection

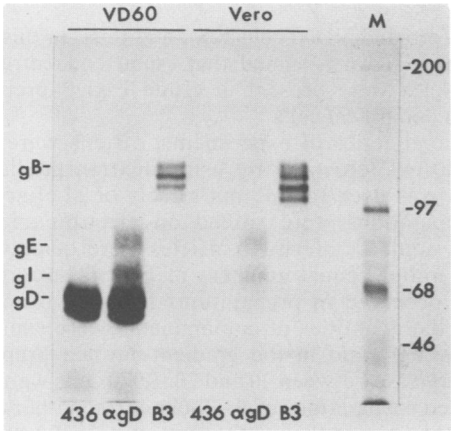

FIG. 2. Expression of gD and gI in F-US6kan-infected cells. VD60 or Vero cells were infected with F-US6kan and labeled with [^{35}S]methionine from 3 h until 8 h postinfection. Cell extracts were made and mixed with monoclonal antibody II-436, specific for gD, a rabbit polyclonal serum specific for gD (αgD) (10), or monoclonal antibody 15βB3 (B3), which is specific for gB (21), and protein A-Sepharose. Note that the rabbit polyclonal serum αgD also precipitated gI and gE because these polypeptides form a complex which has IgG Fc receptor activity which binds rabbit IgG well but mouse IgG poorly (20). Precipitated polypeptides were eluted and electrophoresed through an 8.5% *N,N'*-diallytartardiamide cross-linked polyacrylamide gel. The pattern of multiple bands observed with antibody 15βB3 is most probably due to proteolysis in the cell extract, a common feature with Vero cells (42). The positions of the mature forms of gB, gD, gE, and gI as well as molecular size markers (M) of 200, 97, 68, and 46 kilodaltons are indicated.

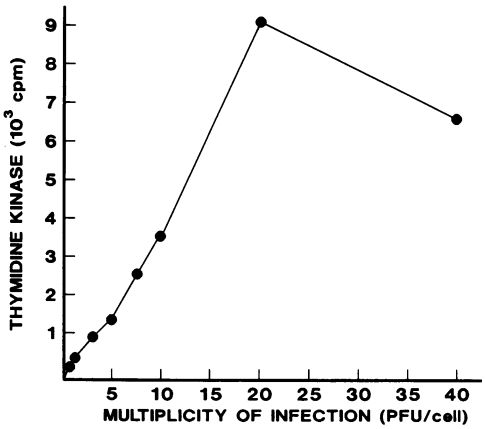

FIG. 3. TK expression versus multiplicity of infection with HSV-1. R970 cells were infected with HSV-1 by using various multiplicities of infection at 4°C. After 2 h, the virus was removed, and the cells were washed and shifted to 37°C for 2 h. The cells were labeled with [^{35}S]methionine for 4 h, and cell extracts were then made. TK was immunoprecipitated by using a rabbit polyclonal serum specific for TK and further purified by electrophoresis in an SDS-polyacrylamide gel. Bands corresponding to TK were excised from the gel and counted in a scintillation counter.

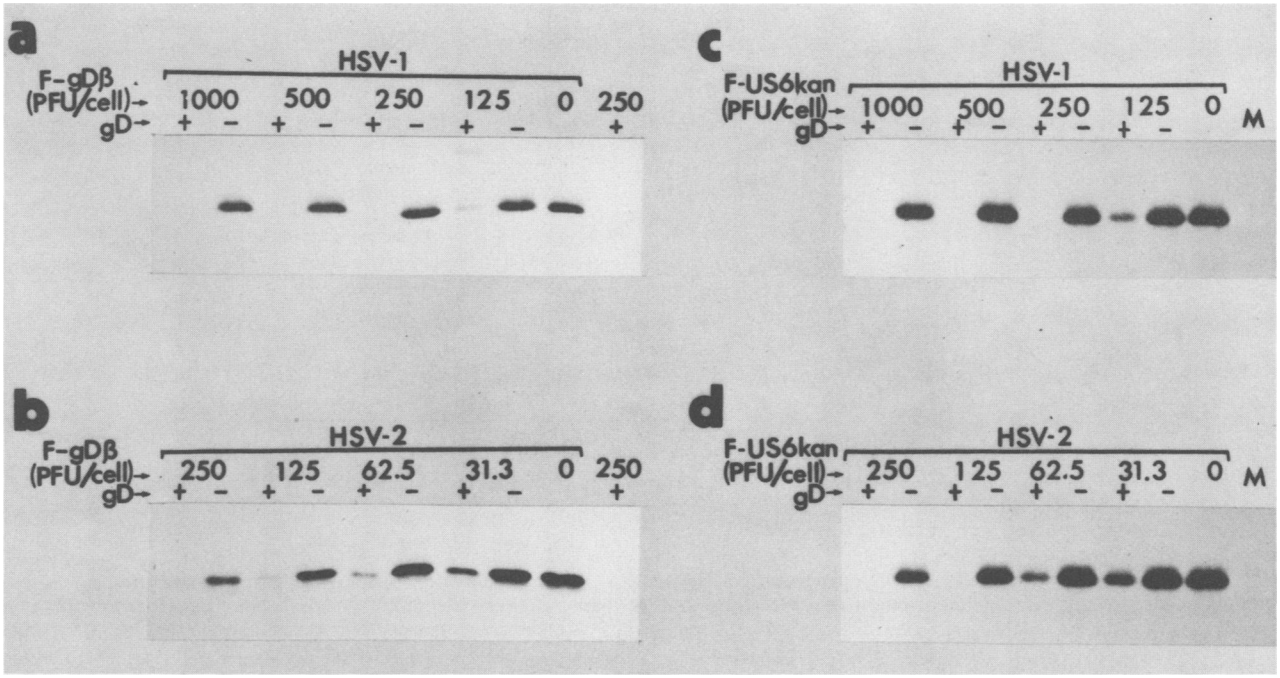

FIG. 4. TK expression in cells pretreated with UV-inactivated F-gDβ or F-US6kan virions with or without gD. Human R970 cells growing in 24-well dishes were treated at 4°C for 2 h with crude stocks of UV-inactivated F-gDβ (a and b) or gradient-purified F-US6kan virions (c and d) derived from VD60 cells (+) or Vero cells (−). The concentrations of UV-inactivated F-gDβ or F-US6kan per cell are indicated along the top of the panels and were calculated by determining the number of PFU in the F-gDβ(VD60) or F-US6kan(VD60) preparations before UV inactivation. The UV-inactivated virus was removed, and the cells were incubated with infectious HSV-1 (a and c) or HSV-2 (b and d) at 4°C. In some wells (far right lanes of panels a and b), the cells were treated with UV-inactivated F-gDβ(VD60) and not infected with HSV-1 or HSV-2 to verify inactivation of the F-gDβ preparations. Some wells of cells (M in panels c and d) were not treated with UV-inactivated virus and not infected. The cells were washed, shifted to 37°C, and then labeled with [^{35}S]methionine. After 4 h, detergent extracts of the cells were made and TK was immunoprecipitated by using a polyclonal serum.

by wild-type HSV. In other experiments, we measured the expression of ICP6, an immediate-early gene product, and two delayed-early polypeptides, gD and gB. The pattern of synthesis of these proteins closely paralleled that of TK (results not shown).

In the first series of experiments, human R970 cells were treated at 4°C with crude extracts of F-gDβ-infected VD60 [F-gDβ(VD60)] or Vero [F-gDβ(Vero)] cells which had been UV inactivated. The UV-inactivated viruses were removed, and the cells were subsequently infected with HSV-1 or HSV-2 at 4°C. The cells were then washed, shifted to 37°C, and labeled with [^{35}S]methionine. Cells pretreated with UV-inactivated F-gDβ(VD60) (virus particles containing gD) at concentrations of 1,000, 500, or 250 PFU per cell showed no evidence of infection with HSV-1, and TK was not expressed at detectable levels (Fig. 4a). In contrast, cells pretreated with a similar quantity of UV-inactivated F-gDβ(Vero) (virus particles lacking gD) displayed virus-induced cytopathic effect similar to infected controls and expressed TK. A reduction of 15 to 30% in TK expression was observed with the highest concentration (corresponding to 1,000 PFU per cell) of F-gDβ(Vero) relative to untreated controls. Cells pretreated with lower concentrations (125 PFU per cell) of F-gDβ(VD60) showed some virus-induced cytopathic effect and expressed low levels of TK, suggesting that the block to infection was not complete. It should be noted that we did not quantitate the numbers of infectious particles in preparations of F-gDβ(Vero) because these viruses are unable to initiate an infection on VD60 cells. However, equal numbers of F-gDβ virus particles are produced in Vero and VD60 cells (23), and thus, in this first set of experiments, we assumed that equal concentrations of virus particles were present in crude F-gDβ preparations from Vero and VD60 cells.

In a second series of experiments, virions were purified from VD60 or Vero cells by using dextran gradients and F-US6kan was used to rule out effects of gI. Each of the virion preparations were spread on electron micrograph grids, and numbers of virus particles were quantitated. In each preparation, equal numbers of F-US6kan virus particles were observed in preparations from Vero and VD60 cells, and the quantities of contaminants were similar. The particle-to-PFU ratio in the gradient-purified preparations was in every case between 20 and 30. R970 cells were treated with purified preparations of F-US6kan from either VD60 or Vero cells at 4°C and then with infectious HSV-1 at 4°C and shifted to 37°C before being labeled with [^{35}S]methionine. The results of these experiments were virtually identical to those involving crude virus stocks of F-gDβ. Cells pretreated with UV-inactivated F-US6kan(VD60) containing gD at concentrations corresponding to 250 PFU per cell (5×10^3 to 7.5×10^3 particles per cell) were not infected by HSV-1, whereas cells pretreated with similar quantities of UV-inactivated F-US6kan(Vero) lacking gD were infected (Fig. 4c). In addition, these observations were not limited to HSV-1 strain F because cells pretreated with F-US6kan(VD60) but not cells treated with F-US6kan(Vero) were resistant to infection with HSV-1 strains HFEM and KOS (results not shown).

The results involving preadsorption with UV-inactivated

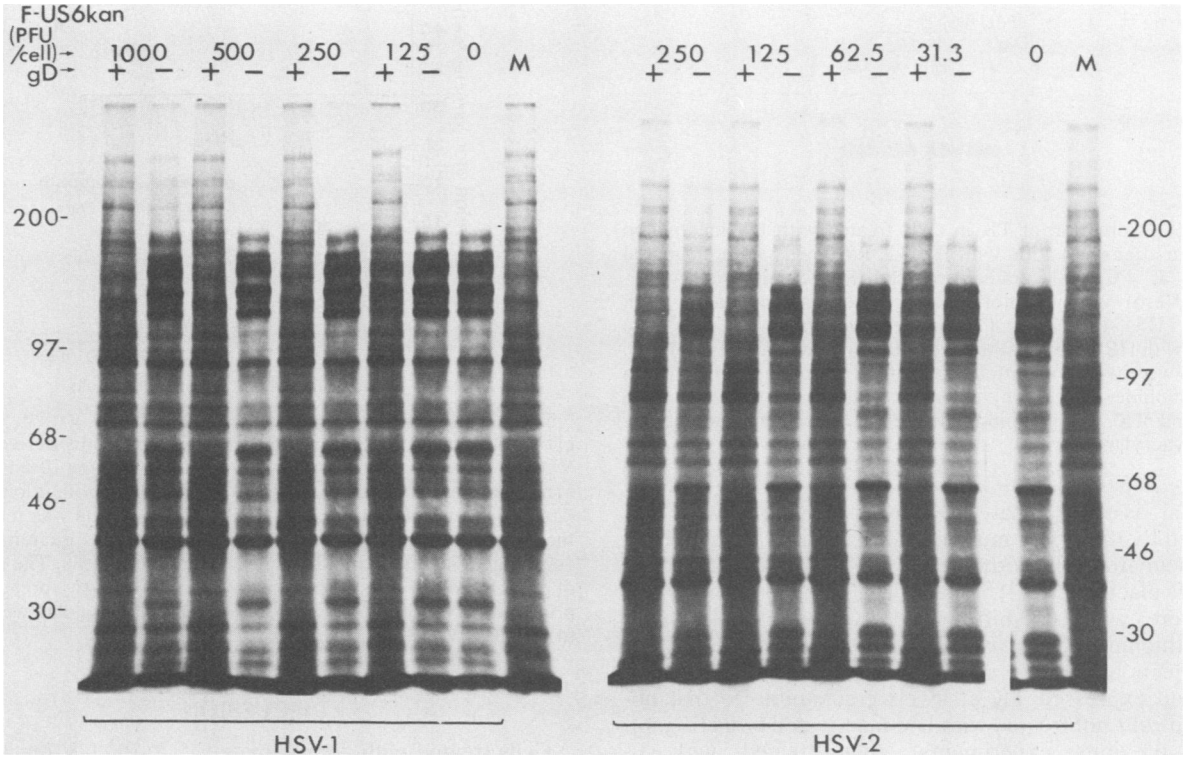

FIG. 5. Expression of viral and cellular polypeptides in cells pretreated with UV-inactivated F-US6kan and then infected with HSV-1 or HSV-2. R970 cells were treated at 4°C for 2 h with gradient-purified F-US6kan(VD60) (+) or F-US6kan(Vero) (−), using virus concentrations indicated along the top of the figure; the cells were then infected at 4°C with HSV-1 (left panel) or HSV-2 (right panel). Lanes 0 represent extracts from cells not treated with UV-inactivated virus but infected with HSV-1 or HSV-2, and lanes M represent extracts of cells not treated with UV-inactivated virus and not infected with HSV-1 or HSV-2. The cells were washed, incubated for 2 h at 37°C, and then labeled with [^{35}S]methionine for 4 h at 37°C. Cell extracts were mixed with SDS and β-mercaptoethanol, boiled, and electrophoresed in SDS-polyacrylamide gels. Molecular size markers of 200, 97, 68, 46, and 30 kilodaltons are indicated.

F-gDβ or F-US6kan and infection with HSV-2 were somewhat different. Cells pretreated with UV-inactivated F-gDβ(VD60) or F-US6kan(VD60) at concentrations corresponding to 250 PFU per cell (5×10^3 to 7.5×10^3 particles per cell) were unable to support the replication of HSV-2; however, a similar quantity of F-gDβ(Vero) or F-US6kan(Vero) also significantly inhibited HSV-2 replication (Fig. 4b and d). When lower concentrations of UV-inactivated F-gDβ(Vero) or F-US6kan(Vero) (corresponding to 125, 62.5, and 31.3 PFU per cell) were used, the inhibition of HSV-2 replication was gradually relaxed. For example, cells pretreated with 125 or 62.5 PFU per cell (1.3×10^3 to 3.7×10^3 particles per cell) of F-US6kan(VD60) expressed low levels of TK, whereas similar quantities of F-US6kan(Vero) had little effect on TK expression. Although the results were less pronounced than with HSV-1, it was obvious that particles containing gD [F-gDβ(VD60) or F-US6kan(VD60)] were more effective in blocking the replication of HSV-2 than were particles lacking gD [F-gD(Vero) or F-US6kan(Vero)]. Similar results were obtained with HSV-2 strains 333 and HG52 (data not shown).

The expression of viral and cellular polypeptides in cells pretreated with either F-US6kan(VD60) or F-US6kan(Vero) and then infected with HSV-1 or HSV-2 was also examined. Cells pretreated with UV-inactivated F-US6kan(VD60) at concentrations corresponding to 250 PFU per cell (5×10^3 to 7.5×10^3 particles per cell) and then infected with HSV-1 displayed a pattern of proteins similar to that observed in cells left uninfected (Fig. 5). In contrast, cells pretreated with an identical number of UV-inactivated F-US6kan(Vero) particles and infected with HSV-1 expressed mainly viral polypeptides, and cellular polypeptide synthesis was inhibited. Similarly, cells pretreated with F-US6kan(VD60) (62.5 PFU per cell) and infected with HSV-2 expressed cellular polypeptides, whereas cells pretreated with a similar quantity of F-US6kan(Vero) expressed HSV-2 polypeptides. Since no viral polypeptides were synthesized in cells pretreated with UV-inactivated virions containing gD and cellular proteins were synthesized at normal levels, it appears likely that virus entry or some other early stage of virus replication is inhibited.

Cells incubated with HSV-1 before treatment with UV-inactivated virions are infected. One hypothesis that could explain inhibitory effects of UV-inactivated virus particles on virus replication is that UV-inactivated virus particles enter cells along with infectious HSV-1 and inhibit intracellular stages of virus replication. Virus particles containing gD, i.e., F-US6kan(VD60), can enter cells, whereas particles lacking gD, i.e., F-US6kan(Vero), do not enter cells and would thus be unable to inhibit intracellular stages of replication. If UV-inactivated particles containing gD inhibit infectious HSV after both have entered cells, then the replication of infectious HSV already bound to cells should be inhibited by subsequent treatment of the cells with UV-inactivated virus. Under these conditions, both UV-inactivated and infectious virions would be expected to enter

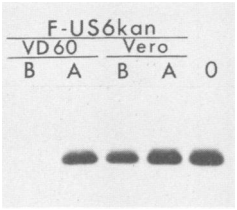

FIG. 6. Expression of TK in cells treated with UV-inactivated F-US6kan after incubation with HSV-1. R970 cells were incubated for 2 h at 4°C with gradient-purified F-US6kan(VD60) or F-US6kan(Vero) virions before (B) or after (A) incubation with infectious HSV-1 for 2 h at 4°C. In one case (lane 0), the cells were infected with HSV-1 but not treated with UV-inactivated F-US6kan. The cells were washed, shifted to 37°C for 2 h, and then labeled with [^{35}S]methionine. After 4 h, detergent extracts of the cells were made, and TK was immunoprecipitated and electrophoresed in SDS-polyacrylamide gels.

the cells, assuming that the infectious virions were not displaced by the higher multiplicity of UV-inactivated virus.

We incubated cells with infectious HSV-1 at 4°C and then with UV-inactivated F-US6kan(VD60) virions at 4°C. The cells were washed, shifted to 37°C, and labeled with [^{35}S]methionine. Cells treated with UV-inactivated virus after infectious HSV-1 displayed typical viral cytopathic effect and expressed TK at levels comparable to that observed in cells not treated with UV-inactivated virus (Fig. 6). As in previous experiments, cells treated with F-US6kan(VD60) before incubation with infectious HSV-1 were not infected. F-US6kan(Vero) particles had no effect on HSV-1 replication whether added before or after infectious virus. Therefore, UV-inactivated virions possessing gD, which can enter cells, apparently do not inhibit intracellular stages of virus replication. Thus, the inhibitory effects of F-US6kan(VD60) added to cells before infectious virus are mediated at the cell surface. In addition, it appears that high multiplicities of virus particles containing gD do not displace infectious HSV-1 already bound to cells.

Adsorption of virus particles lacking gD to cells. We previously concluded that the kinetics of virus adsorption to cells are not affected by the absence of gD in the virion envelope (23). However, to determine how efficiently virus particles lacking gD could absorb to cells when the cells are incubated with high concentrations (>5 × 10^3 particles per cell) of virus and to quantitate cell surface adsorption sites for HSV-1, [^{35}S]methionine-labeled virions were purified by using dextran gradients. In these experiments, radiolabeled virions were incubated with cells at 4°C, although in other experiments, we found that adsorption of virus to cells at 37°C was often 15 to 40% higher than at 4°C (results not shown). However, experiments performed at 37°C probably measure processes other than the initial interactions of virus particles with the cell surface. The adsorption profiles of F-US6kan(VD60) and F-US6kan(Vero) to human R970 cells at 4°C were very similar (Fig. 7). At concentrations of virus below 1 × 10^4 particles per cell (400 PFU per cell), approximately 25% of the labeled virus bound to cells; however, as the concentration of virus increased to 4.5 × 10^5 particles per cell (18,000 PFU per cell), binding gradually dropped to approximately 10% of the input. We were unable to prepare sufficient quantities of virus to saturate virus adsorption to cells. Therefore, there are at least 4.0 × 10^4 sites to which HSV-1 can adsorb on the surfaces of these human cells at 4°C, and the absence of gD in the virion envelope does not affect this process.

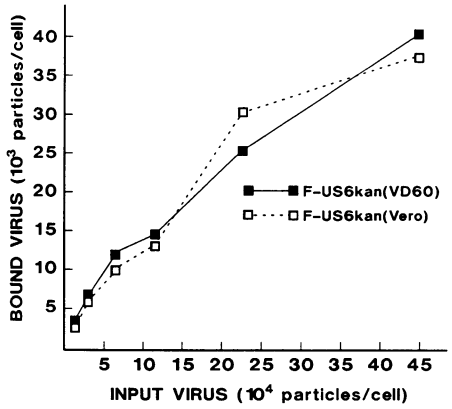

FIG. 7. Adsorption to cells of F-US6kan virions with or without gD. Gradient-purified, radiolabeled F-US6kan virions derived from VD60 or Vero cells were diluted in α-MEM containing 10% FCS and incubated with R970 cell monolayers in 96-well microdilution plates. After adsorption for 3.5 h at 4°C, the unbound virus was removed, the cells were washed twice, and bound virus was quantitated by counting radioactivity in detergent extracts of the cells. The number of particles in the virus preparations was calculated by spreading the preparations on electron microscope grids, and these data were used to calculate virus particles bound per cell.

DISCUSSION

Cells treated with approximately 5 × 10^3 UV-inactivated HSV-1 particles per cell are resistant to infection with HSV-1. This finding is, perhaps, not surprising as others have reported similar results (2, 39, 40). For example, Addison et al. (2) pretreated cells with 4 × 10^3 particles per cell of an HSV-1 mutant which is apparently unable to enter cells and found that the cells were resistant to infection with HSV-1. What is more interesting is the observation that UV-inactivated HSV-1 particles lacking gD are unable to inhibit subsequent infection by HSV-1, suggesting a role for gD in this process. The most likely explanation for this result, especially in light of the essential role of the membrane glycoprotein gD in this process, is that particles containing gD block receptors which are essential for virus entry into cells. Virus particles lacking gD are unable to interact with these receptors and cannot block infection by HSV-1. If this interpretation is correct, then specific cell surface receptors for HSV-1 are quite limited in number. Apparently, there are less than 2 × 10^3 receptor sites per cell because, although cells treated with 5 × 10^3 particles were resistant to HSV-1 infection, only 20 to 25% of the virus particles in these preparations adsorbed to cells.

However, alternative conclusions can also be drawn from our data. We showed previously that virus particles lacking gD are unable to enter cells, whereas those containing gD can enter cells and initiate replication (23). It is possible that UV-inactivated virions containing gD inhibit an early stage of HSV replication other than virus entry into cells. For example, UV-inactivated virus particles containing gD may enter cells and inhibit entry of viral DNA into the nucleus or immediate early gene expression. While we have not totally excluded the possibility that intracellular stages of virus replication are inhibited by UV-inactivated virus particles, the results of two types of experiments suggest that the inhibition is mediated at the plasma membrane. First, cells treated with gradient-purified F-US6kan(VD60) expressed host cell proteins at levels similar to those found in untreated cells, suggesting that there was no generalized toxicity to the

cells. Second, if adsorption of infectious HSV-1 preceded treatment with UV-inactivated F-US6kan(VD60), there was no inhibition of virus replication. In this case, UV-inactivated virus particles containing gD and infectious HSV-1 should both enter cells after the cells were warmed, as would also be the case with cells pretreated with UV-inactivated F-US6kan(VD60) particles. Although large numbers of virions apparently enter the cells, virus replication was only inhibited when the cells were pretreated with UV-inactivated virions containing gD. This result supports the hypothesis that virus particles containing gD can bind to and block cell surface receptors required for virus entry into cells. In addition, this observation suggests that infectious virus particles bound to the surfaces of cells are not displaced by a 25-fold excess of UV-inactivated virions even though the virus particles contain gD.

In contrast to several earlier reports (2, 39, 40), we found that cells preadsorbed with UV-inactivated HSV-1 were resistant to infection with HSV-2 as well as with HSV-1. It is difficult to compare our results directly with those of Valhne et al. (39, 40) because these authors measured virus adsorption to cells which had been trypsinized and pretreated six times with UV-inactivated HSV-1 or HSV-2 at 37°C. The results of Addison et al. (2), who found that cells pretreated with an HSV-1 mutant which is unable to enter cells are resistant to infection with HSV-1 but not HSV-2, are difficult to reconcile with our results except that in their experiments the cells were incubated with viruses at 39°C. However, the hypotheses that HSV-1 and HSV-2 use similar or identical cell surface receptors during the entry process and that gD interacts with these receptors are supported by the observations of Campadelli-Fiume et al. (6). These investigators found that cells expressing HSV-1 gD were resistant to HSV-1 and HSV-2. Presumably, gD in the plasma membrane of transfected cells is able to interact with virus receptors. We do not fully understand our observation that HSV-2 replication was inhibited by using lower concentrations of UV-inactivated virus than were necessary to inhibit HSV-1 replication. This finding may or may not be related to the fact that the particle-to-PFU ratio of our HSV-2 stocks is 3- to 10-fold higher than that of our HSV-1 stocks (D. C. Johnson, unpublished results).

Although these and other studies have suggested a role for gD in virus penetration into cells, we could find no evidence that gD is necessary for the initial attachment or adsorption of virus to cells. Large quantities ($>4.0 \times 10^4$ particles per cell) of HSV-1 with or without gD could adsorb to cells. These results suggest a working model for the role of gD in the attachment and penetration of HSV into cells. At this point, the model lacks many important details; however, it may be useful in the design of further experiments. In this model (Fig. 8), HSV particles can adsorb to a large number of sites on the cell surface, possibly involving more than a single host plasma membrane molecule. Adsorption of virions to the cell surface most probably involves several of the virion envelope glycoproteins. A small fraction of the adsorbed virus particles binds via gD molecules to specific cell surface receptors. Alternatively, virus particles adsorbed to the cell surface at other sites may diffuse in the plane of the membrane and subsequently interact with gD-specific receptors. Only virions bound to these gD-specific receptors are able to enter cells by a fusion event between the virion envelope and the plasma membrane. Virus particles lacking gD are unable to bind to the receptors and allow entry of superinfecting HSV-1. Although, we have no direct evidence at the present time that gD is the receptor-binding polypeptide, the evidence presented here and elsewhere suggests that this is likely.

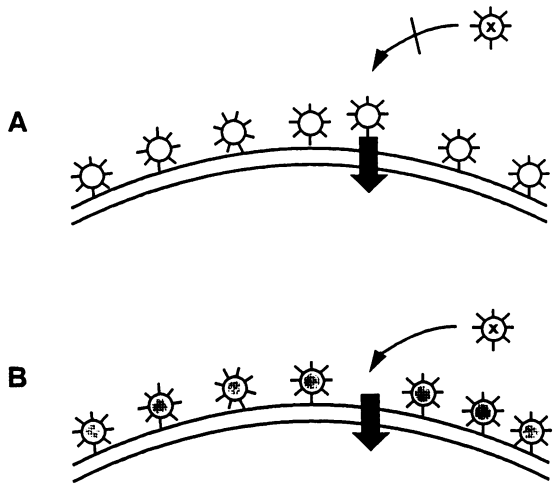

FIG. 8. Model describing the role of gD in HSV adsorption and entry into cells. (A) Virus particles containing gD, i.e., F-US6kan virions produced in VD60 cells (open symbols), can adsorb at numerous sites on the surfaces of human cells and via gD molecules to more specialized receptors (large arrows in the plasma membrane). Virus particles which interact with the gD-specific receptors can enter cells, while viruses adsorbed at other sites do not enter the cells directly. Therefore, when cells preadsorbed with UV-inactivated virus particles containing gD are incubated with infectious HSV-1 (symbols with X's), the receptors are occupied and the infectious virus is unable to enter the cells. (B) Virus particles lacking gD, i.e., F-US6kan virions produced on Vero cells (shaded symbols), can also adsorb to the numerous adsorption sites on the cell surface; however, these particles cannot interact with gD-specific receptors. Because these particles do not occupy the receptors, infectious HSV-1 can bind and enter cells.

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