JOURNAL OF VIROLOGY, Oct. 1997, p. 8024–8028 Vol. 71, No. 10
0022-538X/97/$04.00+0

Partial Resistance to gD-Mediated Interference Conferred by Mutations Affecting Herpes Simplex Virus Type 1 gC and gK

PETER E. PERTEL[1,2] AND PATRICIA G. SPEAR[2]*

Division of Infectious Disease, Department of Medicine,[1] and Department of Microbiology-Immunology,[2] Northwestern University Medical School, Chicago, Illinois 60611

Received 28 May 1997/Accepted 30 June 1997

Cells expressing herpes simplex virus (HSV) gD can be resistant to HSV entry as a result of gD-mediated interference. HSV strains differ in sensitivity to this interference, which blocks viral penetration but not binding. Previous studies have shown that mutations or variations in virion-associated gD can confer resistance to gD-mediated interference. Here we show that HSV-1 mutants selected for enhanced ability to bind and penetrate in the presence of inhibitory concentrations of heparin were partially resistant to gD-mediated interference. The resistance was largely due to the presence of two mutations: one in gC (the major heparin-binding glycoprotein) resulting in the absence of gC expression and the other in gK resulting in a syncytial phenotype. The results imply that heparin selected for mutants with altered postbinding requirements for entry. Resistance to gD-mediated interference conferred by mutations affecting gC and gK has not been previously described.

Initial absorption of herpes simplex virus type 1 (HSV-1) to cells is mediated by interaction of a viral glycoprotein, gC (UL44), with cell surface heparan sulfate (18, 20, 41, 44, 50). Glycoprotein B (UL27) mediates initial binding for virus that does not express gC and is also required for subsequent penetration (5, 19, 29, 39). Other viral glycoproteins essential for penetration include gD (US6), gH (UL22), and gL (UL1), and possibly the tegument protein UL25 (1, 15, 28, 38). Penetration of virus may also involve interaction with cell surface heparan sulfate (40). Soluble heparin, which is chemically similar to heparan sulfate (24), can inhibit viral binding and penetration (36, 41, 50). Penetration of HSV into cells proceeds by a series of steps requiring several viral glycoproteins and cell surface molecules, culminating in fusion of the viral envelope with the cell plasma membrane and release of the nucleocapsid into the cell cytoplasm (16, 17, 26, 32, 35, 49).

Recently, a gene was cloned that encodes a cell surface protein capable of mediating HSV entry (31). The protein, designated herpesvirus entry mediator (HVEM), is a member of the tumor necrosis factor-nerve growth factor receptor family and appears to be one of several cellular proteins that can mediate viral entry (31, 46). Expression of HVEM in nonhuman cells resistant to HSV entry confers susceptibility to viral infectivity. In addition, anti-HVEM antibodies inhibit HSV-1 infection of HVEM-expressing Chinese hamster ovary (CHO) cells and of activated human T cells, indicating that HVEM mediates HSV-1 entry into certain types of human cells (31). HVEM interacts directly with gD and can be considered a coreceptor for HSV entry (48). Other cell surface molecules have been shown to interact with gD but either fail to mediate HSV entry or have not yet been identified (3, 4, 22).

Cells that express HSV gD are resistant to infection with wild-type virus as a result of gD-mediated interference (6, 7, 25). Interference has been noted with several cell lines engineered to express gD, including HEp-2 cells, BHK cells, L cells, and HVEM-expressing CHO cells (6, 7, 25, 47). Interference has also been noted with other alphaherpesviruses, including HSV-2, pseudorabies virus, and bovine herpesvirus type 1 (10, 11, 14, 37, 43). Cross interference between alphaherpesviruses can occur but is not always reciprocal (10, 11, 27, 37). Although the mechanism is not fully defined, gD-mediated interference appears to occur after binding at the level of penetration (6, 13, 25). One proposal is that interference involves competition between cell-associated gD and virion-associated gD for a cellular coreceptor for entry (6, 7, 13, 25).

HSV-1 mutants that are resistant to gD-mediated interference have been isolated. Some have been selected for their ability to propagate in cells expressing gD (7, 13). These mutants contain altered forms of gD that can partially or fully account for any noted resistance. Other interference-resistant mutants, again containing altered forms of gD, were selected for resistance to neutralization by a specific anti-gD monoclonal antibody (30). In addition, wild-type strains of HSV can vary in sensitivity to gD-mediated interference, due to polymorphisms in gD and other undefined loci (14). Mutants with alterations in gD that confer resistance to interference have greatly reduced ability to utilize HVEM for entry but can infect human cells via other pathways (13, 31, 47). Moreover, wild-type gD interacts directly with HVEM, while the binding of the mutant forms of gD to HVEM cannot be detected (48). It may be that differences in HSV sensitivity to gD-mediated interference correlate with relative ability to use HVEM and other mediators of entry.

Recently we described the properties of mutants obtained by selection for variants of HSV-1(KOS) (designated KOS) that could bind and penetrate in the presence of heparin (36). The heparin-selected uncloned virus pools contained predominantly syncytial and gC-negative mutants. Syncytial mutants were plaque purified from the uncloned pools and designated HS, for heparin-selected: two were gC positive (HS2 and HS4), and two were gC negative (HS1 and HS3). The gC-negative mutants were significantly more resistant to heparin inhibition than were the gC-positive mutants. For all four plaque-purified mutants, the syncytial phenotype mapped to a missense mutation (A40V) in gK (UL53). The gK mutation did not contribute to heparin resistance of HS1 and HS3; absence of gC

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University Medical School, Ward Bldg., Room 6-241, MC W213, 303 E. Chicago Ave., Chicago, IL 60611. Phone: (312) 503-8230. Fax: (312) 503-1339. E-mail: p-spear@nwu.edu.

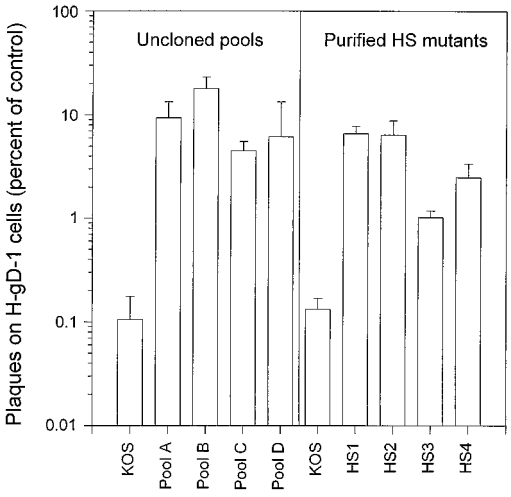

FIG. 1. Resistance of the uncloned virus pools and plaque-purified heparin-selected mutants to gD-mediated interference. Virus diluted in complete phosphate-buffered saline was inoculated onto six-well cultures of H-gD-1 cells or H-control cells for 2 h at 37°C (25). After the inoculum was removed, the cells were overlaid with medium 199 supplemented with 1% heat-inactivated newborn calf serum and 0.1% pooled human gamma globulin (Armour; 199O) and then incubated for 3 days at 37°C in 5% CO_2. Plaques were visualized by an anti-gB immunoassay and counted (20, 21). Results are presented on a logarithmic scale as the number of plaques on H-gD-1 cells as a percentage of the number on H-control cells. The geometric means and standard deviations for three different experiments each done in duplicate are shown for each pool and virus. Differences between KOS and each pool (A, B, C, and D) or mutant (HS1, HS2, HS3, and HS4) were statistically significant (left panel, $P = 0.002$, $P < 0.001$, $P = 0.002$, and $P = 0.012$, respectively; right panel, $P < 0.001$; two-tailed t test).

expression alone was found to be sufficient to confer resistance to heparin inhibition. Because the infectivity of gC-negative virus is reduced (19, 20), the syncytial mutation in gK may have conferred a selective advantage by enhancing the spread of the virus through the cell monolayer by cell fusion.

Interestingly, HSV-1(ANG), a syncytial strain of HSV that was plaque purified under agar medium (33, 34), is resistant to gD-mediated interference because of amino acid substitutions at the same positions in gD as for other interference-resistant mutants (13). Because agar, like heparin, contains sulfated polysaccharides that can inhibit HSV binding (42), this study was undertaken to determine whether the heparin-selected mutants were altered in their ability to infect cells expressing gD.

Resistance to gD-mediated interference. To test the relative susceptibility of the heparin-selected mutants and the uncloned pools to gD-mediated interference, virus was inoculated onto HEp-2 cells that either express HSV-1 gD (H-gD-1 cells) or do not (H-control cells) (25). Shown in Fig. 1 are the number of plaques on H-gD-1 cells as a percentage of the number on H-control cells. All four uncloned virus pools exhibited partial resistance to gD-mediated interference (left panel), as did the four heparin-selected cloned mutants (right panel). The number of plaques formed on H-gD-1 compared to that on control cells was approximately 1 to 10% for the uncloned pools and the four heparin-selected mutants. In contrast, the number of plaques on gD-expressing cells compared to that on control cells obtained with interference-resistant mutants altered in gD was 10 to 100% (7, 13).

To define mutations in the heparin-selected mutants that contribute to the partial resistance to gD-mediated interference, several virus strains were used. HS3 was selected because its syncytial phenotype and heparin resistance were previously shown to be determined entirely by a single amino acid substitution (A40V) in gK and a mutation resulting in absence of gC expression (36). A gC-positive syncytial recombinant virus, designated SyngK, was constructed by transfer of the HS3 gK gene to KOS (36). The gC-negative recombinant virus HSV-1(KOS)ΔgC2-3 (designated ΔgC2-3), in which the gC gene is replaced by the *Escherichia coli lacZ* gene, has been previously described (19). In order to construct a virus strain carrying both mutations known to be present in HS3, but lacking other mutations that might also be present, a gC-negative syncytial recombinant (designated ΔgC2-3syngK) was isolated from the progeny of cells coinfected with SyngK and ΔgC2-3 (36). The genotypes and phenotypes of each recombinant were confirmed by detection of characteristic restriction endonuclease sites in amplified copies of the gK genes and by monitoring of gC and β-galactosidase expression (data not shown). The gD-negative mutant HSV-1(KOS)gDβ (designated KOSgDβ) (13) was used as the recipient in marker transfer experiments. Finally, the interference-resistant mutant HSV-1(KOS)Rid1 (designated Rid1) was used as the donor for a mutant form of gD that confers a high level of resistance to gD-mediated interference (13).

Absence of interference resistance mutations in HS3 gD. Comparison of the ability of gD genes from HS3, KOS, ΔgC2-3, and Rid1 to transfer to recombinant viruses resistance to gD-mediated interference tested the possibility that mutations in HS3 gD were responsible for the noted resistance. The gD gene from Rid1 served as a positive control, while the gD gene from KOS, which is extremely sensitive to gD-mediated interference (14), served as a negative control. Vero cells were transfected with genomic DNA from KOSgDβ along with gD genes obtained by PCR amplification from each virus of interest (14). Because gD is essential for HSV propagation, progeny capable of forming plaques on noncomplementing cells will be recombinant virus carrying the gD gene of the donor strain. The titer of recombinant virus produced by the transfected cells on H-gD-1 cells and H-control cells was determined. Figure 2 shows that only the Rid1 gD gene was able to confer resistance to gD-mediated interference, whereas the HS3 and ΔgC2-3 gD genes gave results indistinguishable from those obtained with the KOS gD gene. Results similar to those obtained with the HS3 gD gene were also obtained with the HS1 gD gene (data not shown).

To exclude the possibility of mutations in the gD gene that can confer partial resistance to interference only when expressed with other mutations, such as those altering gC or gK, the gD gene from HS3 was sequenced. The gD gene was amplified by PCR in two separate reactions, and both products were cloned into pGEM4 (Promega). The nucleotide sequence was determined with an ABI Prism 377 DNA sequencer (University of Chicago Cancer Research Center DNA Sequencing Facility). The nucleotide sequence of HS3 gD did not differ from the published KOS gD sequence (data not shown).

Because the amount of gD incorporated into the viral envelope may influence susceptibility to gD-mediated interference, the relative amounts of gD present in purified KOS and HS3 virions were assessed. Shown in Table 1 are the relative amounts of gD and the major capsid protein VP5 present in preparations of KOS and HS3 virions as assessed by a bioimager as follows. Basically, purified KOS and HS3 (9) were solubilized in sodium dodecyl sulfate sample buffer, boiled for 5 min, and then loaded onto a sodium dodecyl sulfate–7% polyacrylamide gel (*N*,*N*′-methylene-bisacrylamide) (Bio-Rad laboratories). After electrophoresis, the proteins were transferred to nitrocellulose (Schleicher & Schuell) with a Trans-Blot electrophoretic transfer cell apparatus (Bio-Rad labora-

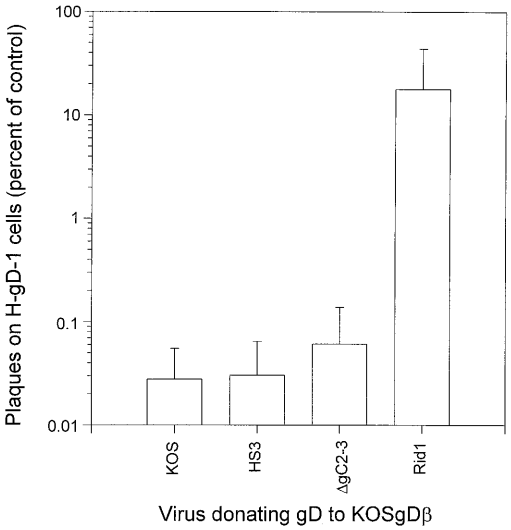

FIG. 2. Absence of gD mutations that could confer resistance to gD-mediated interference. Subconfluent Vero cells were transfected with Lipofectamine reagent (Gibco BRL) with 1 μg of genomic KOSgDβ DNA and 1 μg of the gD gene obtained by PCR amplification from each virus (14). Genomic viral DNA was purified from infected cell lysates as previously described (36). PCR was performed with Vent polymerase (New England Biolabs) and purified viral DNA. The gD gene along with flanking sequences was amplified with the sense primer gD1-5 (5′-GGAGTTGTTCGGTCATAAGCTTCAGC-3′) and the antisense primer gD1-7 (5′-GACTTATCGACTGTCCGCCTTTC-3′) (13), obtained from the Northwestern University Biotechnology Facility. After the transfection mixture was removed, the cells were overlaid with medium 199 supplemented with 1% heat inactivated newborn calf serum (199V) and then were incubated for 48 h at 37°C in 5% CO_2. The cells were then harvested and lysed to release recombinant progeny. Three separate PCR amplifications and transfections were done, generating three recombinant stocks that each contain the gD gene of interest. The titer of each recombinant stock on H-gD-1 cells and H-control cells was subsequently determined. Results are presented on a logarithmic scale as the number of plaques on H-gD-1 cells as a percentage of the number on H-control cells. The geometric means and standard deviations for three different experiments each done in duplicate are shown. Differences between the recombinant stocks containing the gK gene from KOS and the genes from HS3 and ΔgC2-3 were not statistically significant ($P = 0.933$ and 0.637, respectively; two-tailed t test). Differences between the recombinant stocks containing the gK gene from Rid1 and the genes from KOS, HS3, and ΔgC2-3 were all statistically significant ($P = 0.008$, 0.009, and 0.015, respectively).

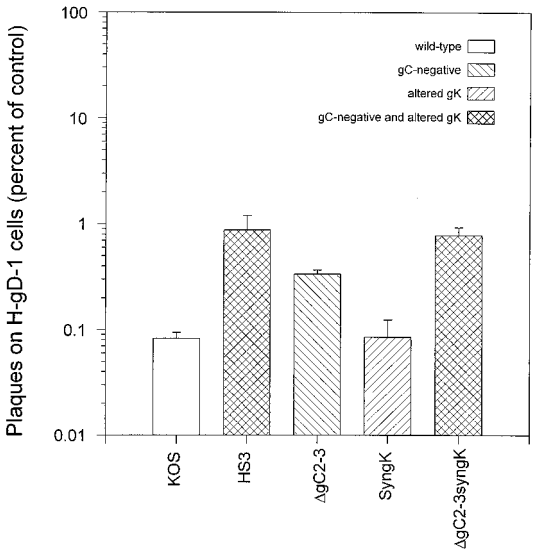

FIG. 3. Resistance of recombinant viruses to gD-mediated interference. The titer of virus diluted in phosphate-buffered saline on H-gD-1 cells and H-control cells was determined as described in the legend to Fig. 1. Results are presented on a logarithmic scale as the number of plaques on H-gD-1 cells as a percentage of the number on H-control cells. The geometric means and standard deviations for three different experiments each done in duplicate are shown for each pool and virus. Differences between KOS and HS3, ΔgC2-3, and ΔgC2-3syngK were all statistically significant ($P < 0.001$; two-tailed t test); the difference between KOS and SyngK was not ($P = 0.928$). Differences between HS3 and ΔgC2-3 and between HS3 and SyngK were both statistically significant ($P = 0.017$ and 0.002, respectively); the difference between HS3 and ΔgC2-3syngK was not ($P = 0.768$).

tories). The nitrocellulose blot was incubated in blocking buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 0.2% Tween 20, 0.02% thimerosal, 5% milk) and then was incubated with a pool of polyclonal rabbit antiserum at 4°C followed by ^{35}S-labeled anti-rabbit immunoglobulin (Amersham) at room temperature. Rabbit anti-gD antibody R-7 (diluted 1:1,000) and anti-VP5 antibody NC-1 (diluted 1:2,000) were used (12, 23). Reactive bands were visualized, and the photostimulated luminescence value was quantified with a FUJIX BAS 2000 bioimager (Fuji). No significant difference in the gD/VP5 ratios for KOS and HS3 was noted.

TABLE 1. Relative amounts of gD and VP5 present in purified KOS and HS3 virions[a]

Virus	Dilution	Photostimulated luminescence		gD/VP5 ratio
		VP5	gD	
KOS	None	3,970	505	0.13
	1:2	2,350	343	0.15
	1:4	1,100	183	0.17
	1:8	634	106	0.17
HS3	None	2,240	291	0.13
	1:2	1,330	149	0.11
	1:4	636	83	0.13
	1:8	328	36	0.11

[a] For details, see the text. Photostimulated luminescence values have been corrected for background radioactivity.

Mutations responsible for resistance to gD-mediated interference. To evaluate whether lack of gC expression or a syncytial mutation could confer partial resistance to gD-mediated interference, recombinants expressing these phenotypes either alone or together were studied. The titers of the engineered recombinants along with KOS and HS3 on H-gD-1 and H-control cells were determined. As shown in Fig. 3, both HS3 and ΔgC2-3syngK were approximately 10-fold less sensitive to gD-mediated interference than KOS, with no difference noted between HS3 and ΔgC2-3syngK. Interestingly, the engineered gC-negative virus, ΔgC2-3, was approximately threefold less sensitive to gD-mediated interference than was KOS. No difference was noted between KOS and SyngK. The revertants for SyngK (36) and ΔgC2-3 (19) also did not differ from KOS (data not shown). Results similar to those shown in Fig. 3 were obtained with a second engineered gC-negative virus, HSV-1(KOS)ΔgC6, and its revertant (45) and a double recombinant constructed by transfer of the gK allele from HS3 to ΔgC6 (data not shown). Thus, the lack of gC expression conferred slight resistance to gD-mediated interference, while the syncytial mutation in gK did not. Combination of the two mutations resulted in a recombinant virus that was as resistant to interference as HS3. The resistance to gD-mediated interference conferred by these two mutations, however, is less than that conferred by single mutations in HSV-1 gD.

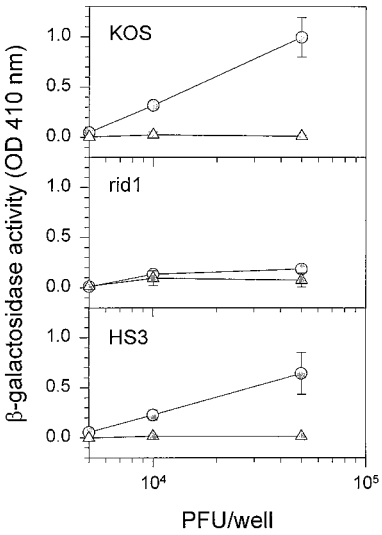

FIG. 4. HVEM-mediated entry of HSV into CHO cells. Subconfluent CHO-IEβ8 cells were transfected with Lipofectamine reagent (Gibco BRL) with 1.5 μg of a plasmid (pBEC10) containing HVEM downstream from a constitutive cytomegalovirus immediate-early promoter (31). The negative control consisted of 1.5 μg of the plasmid vector (pcDNA3) without the insert. After 24 h, the cells were replated in 96-well plates at a concentration of about 2×10^4 to 4×10^4 cells/well and then incubated for 24 h at 37°C in 5% CO_2. The cells were washed and then inoculated with 50 μl of virus diluted in PBS containing 0.1% glucose and 1% calf serum (Sigma). The number of PFU added to each well was based on titers on Vero cells and was confirmed in each experiment by simultaneous plaquing of each virus on Vero cells. After incubation for 6 h at 37°C, 100 μl of phosphate-buffered saline containing 0.5% Nonidet P-40 and 3 mg of the β-galactosidase substrate *o*-nitrophenol β-D-glucopyranoside per ml was added to each well. Optical density (OD) readings at 410 nm were obtained every hour with a plate spectrophotometer (Spectromax 250). The results shown are mean optical density readings and standard deviations obtained at 4 h, at which time product accumulation was proportional to time, for triplicate determinations at concentrations of input virus within the linear range of dose-response curves. The circles represent readings obtained from cells transfected with HVEM, and the triangles represent readings from cells transfected with the vector control. Similar results were obtained in a second set of experiments done independently (data not shown).

HVEM-mediated entry of HS3. Mutants with alterations in gD that confer resistance to interference have greatly reduced ability to utilize HVEM for entry but can infect human cells via other pathways (13, 31, 47). Because HS3 is partially resistant to gD-mediated interference, the ability of HVEM to mediate entry of HS3 was tested. CHO cells allow KOS to bind efficiently but are not susceptible to subsequent penetration (41). CHO-IEβ8 cells were transfected with pBEC10, which expresses HVEM, and then infected with HS3 as previously described (31). CHO-IEβ8 cells carry the *E. coli lacZ* gene downstream from an HSV-1 immediate-early promoter and express β-galactosidase when induced by the HSV-1 transinducer VP16 (31). VP16 is released along with the viral nucleocapsid when HSV enters a cell and does not require any viral gene expression to function (2, 8). Measurement of β-galactosidase activity induced by VP16, therefore, can be used to quantitate initial HSV entry. As shown in Fig. 4, both KOS and HS3 could utilize HVEM to enter into CHO cells, while Rid1 could not. Results similar to those noted for KOS and HS3 were obtained with ΔgC2-3, SyngK, and ΔgC2-3syngK (data not shown). Thus, the partial resistance to interference noted for HS3 does not correlate with an inability to use HVEM to mediate entry.

In summary, HSV-1 mutants selected for resistance to the inhibitory effects of heparin on virus binding and penetration were also partially resistant to gD-mediated interference. For at least one mutant, HS3, a syncytial mutation in gK and lack of gC expression were sufficient to account for the noted resistance. In addition, lack of gC expression alone resulted in partial resistance to gD-mediated interference. These are previously undescribed phenotypes of mutations affecting gC and gK. These results are consistent with those of a prior study showing that viral genes in addition to that encoding gD can influence resistance to gD-mediated interference (14). Mutations in the gD gene that confer resistance to interference reduce the ability of virus to utilize HVEM as a coreceptor for entry (31). Mutations in the gC and gK genes that confer resistance do not but may instead alter usage of other coreceptors for HSV entry. The heparin-selected mutants may prove useful in defining patterns of coreceptor usage.

We thank N. Susmarski for the preparation of cell cultures. HSV-1(KOS)ΔgC6 and HSV-1(KOS)ΔgC2-3 and their revertants were gifts from C. Brandt (University of Wisconsin). Antibodies R-7 and NC-1 were gifts from G. Cohen and R. Eisenberg (University of Pennsylvania).

These studies were supported by a grant from the National Institutes of Health (R01 AI 36293-03) and a fellowship from the Howard Hughes Medical Institute, Chevy Chase, Md. (P.E.P.)

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