JOURNAL OF VIROLOGY, July 1998, p. 5802–5810 Vol. 72, No. 7
0022-538X/98/$04.00+0

HveA (Herpesvirus Entry Mediator A), a Coreceptor for Herpes Simplex Virus Entry, also Participates in Virus-Induced Cell Fusion

TRACY TERRY-ALLISON,[1] REBECCA I. MONTGOMERY,[1] J. CHARLES WHITBECK,[2,3,4] RULIANG XU,[2,3,4] GARY H. COHEN,[2,3] ROSELYN J. EISENBERG,[3,4] AND PATRICIA G. SPEAR[1]*

Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611,[1] and School of Dental Medicine,[2] Center for Oral Health Research,[3] and School of Veterinary Medicine,[4] University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received 24 November 1997/Accepted 7 April 1998

The purpose of this study was to determine whether a cell surface protein that can serve as coreceptor for herpes simplex virus type 1 (HSV-1) entry, herpesvirus entry mediator (previously designated HVEM but renamed HveA), also mediates HSV-1-induced cell-cell fusion. We found that transfection of DNA from KOS-804, a previously described HSV-1 syncytial (Syn) strain whose Syn mutation was mapped to an amino acid substitution in gK, induced numerous large syncytia on HveA-expressing Chinese hamster ovary cells (CHO-HVEM12) but not on control cells (CHO-C8). Antibodies specific for gD as well as for HveA were effective inhibitors of KOS-804-induced fusion, consistent with previously described direct interactions between gD and HveA. Since mutations in gD determine the ability of HSV-1 to utilize HveA for entry, we examined whether the form of virally expressed gD also influenced the ability of HveA to mediate fusion. We produced a recombinant virus carrying the KOS-804 Syn mutation and the KOS-Rid1 gD mutation, which significantly reduces viral entry via HveA, and designated it KOS-SR1. KOS-SR1 DNA had a markedly reduced ability to induce syncytia on CHO-HVEM12 cells and a somewhat enhanced ability to induce syncytia on CHO-C8 cells. These results support previous findings concerning the relative abilities of KOS and KOS-Rid1 to infect CHO-HVEM12 and CHO-C8 cells. Thus, HveA mediates cell-cell fusion as well as viral entry and both activities of HveA are contingent upon the form of gD expressed by the virus.

Herpes simplex virus (HSV)-induced cell-cell fusion requires the concerted actions of several cellular and viral components. The viral components can be divided into two categories: mediators and modulators (for a review, see reference 51). The mediators, so called because their absence blocks or mutes the syncytial (Syn) phenotype, include the glycoproteins gB (UL27) (6, 10, 31), gD (US6), (27, 38), gH-gL (UL22-UL1) (which function as a hetero-oligomer) (8, 14, 22, 44), gM (UL10), gE (US8), and gI (US7) (2, 7, 8) and the membrane protein encoded by gene UL45 (17). The gene products UL20, UL24, gK (UL53), and gB are termed modulators of cell fusion because mutations in any one of the four can confer the Syn phenotype (1, 3, 4, 13, 15, 23, 24, 31, 42, 45, 46). For a strain to be syncytial, it must express gB, gD, gH, and gL and perhaps other functional mediators and also have a Syn mutation in one of the modulator genes (51). Work by Shieh and Spear (48) indicated that cellular factors such as cell surface glycosaminoglycans (GAGs) also play a role in HSV-induced cell-cell fusion. They demonstrated that wild-type, but not GAG-deficient, Chinese hamster ovary (CHO) cells formed syncytia after transfection with DNA from an HSV type 1 (HSV-1) Syn mutant. When soluble heparin was exogenously added the GAG-deficient cells were able to fuse, strongly implicating a role for GAGs in HSV-induced cell fusion.

HSV entry, like fusion, also requires the interaction of multiple viral and cellular components and can be divided into two distinct events: binding and penetration. Viral glycoproteins gC and gB mediate attachment of the virion to the cell surface through their interactions with heparan sulfate chains on cell surface proteoglycans (18, 19, 49, 57). Penetration, which occurs via fusion of the viral envelope with the plasma membrane at neutral pH (56), requires the viral glycoproteins gB, gD, and the hetero-oligomer gH-gL. Virions devoid of any of these glycoproteins bind cells but fail to penetrate (6, 14, 27, 44). Cells devoid of specific cell surface receptors, other than proteoglycans, bind virus but restrict entry (36, 43, 49). Expression of the human cell surface glycoprotein HveA (herpesvirus entry mediator A) can confer susceptibility to HSV-1 infection on normally resistant CHO cells (36). HveA, a member of the tumor necrosis factor receptor family, mediates entry of HSV-1 into a subset of human cell types including T lymphocytes (36). Interestingly, the form of gD expressed by the virus dictates its ability to utilize the HveA entry pathway. HSV-1(KOS)Rid1, which was isolated for its ability to overcome gD-mediated interference and has a mutation in the gD gene (Q27P), is greatly impaired in its ability to utilize the HveA entry pathway (9, 36). Using a soluble form of HveA produced in a baculovirus system, Whitbeck et al. (55) reported that HveA binds to wild-type gD but not Rid1 gD, thus explaining why mutations in gD affect the ability of such viruses to utilize HveA for entry.

The processes of HSV entry and HSV-induced cell fusion involve many of the same viral and cellular components, and both involve membrane fusion. However, several lines of evidence support the position that HSV entry and fusion are distinct processes. First, viruses competent for entry are not automatically competent to induce cell fusion (12). Though syncytium formation is a common event in vivo, clinical isolates

* Corresponding author. Mailing address: Department of Microbiology-Immunology, Northwestern University Medical School, Ward Memorial Building, 303 East Chicago Ave., Chicago, IL 60611-3008. Phone: (312) 503-8230. Fax: (312) 503-1339. E-mail: p-spear@nwu.edu.

TABLE 1. Mapping of 804 gK Syn mutation by marker transfer and marker repair

Virus donating gK DNA fragment[a]	Recipient genomic viral DNA	Frequency of Syn plaques[b] (%)	Frequency of non-Syn plaques[c] (%)
804	KOS	16/2,517 (0.63)	
804	804		0/846 (<0.01)
KOS	KOS	2/2,233 (0.08)	
KOS	804		39/1,554 (2.50)

[a] The DNA fragment used spans amino acids 172 to 305 of the gK ORF.
[b] Number of Syn plaques/total number of plaques.
[c] Number of non-Syn plaques/total number of plaques.

of HSV rarely induce the fusion of cultured cells (20, 52). Nonlethal spontaneous mutations, which can arise during the passage of clinical isolates through cultured cells, confer the Syn phenotype without obvious effects on viral entry (20, 52). Second, susceptibility of cells to HSV infection does not guarantee susceptibility to HSV-induced cell fusion. Some cultured cell lines, while being highly susceptible to infection by HSV, are resistant to HSV-induced cell fusion (5, 26, 28, 51, 52). This finding suggests that cellular factors somehow differentiate between entry and cell fusion, causing cells to succumb to one process while resisting the other. Third, drugs can distinguish between the two events. Cyclosporin A, which selectively inhibits cell fusion induced by some HSV-1 strains, has no effect on entry (32, 54). Finally, several viral genes required for expression of the Syn phenotype play no role in entry. Though the glycoproteins gE, gI, and gM and the membrane protein encoded by the UL45 gene have been reported to contribute toward expression of the Syn phenotype, all are dispensable for entry (8, 29, 30, 53). While these observations clearly accent the differences between the processes of entry and fusion, the mechanisms of membrane fusion in either case remain unknown.

Considering the differences between entry and cell fusion outlined above, a cell surface protein such as HveA, shown to function in entry, may not necessarily serve the same role or even have a role in HSV-1-induced cell fusion. We therefore explored the role of HveA in HSV-1-induced cell fusion, using syncytial viruses that express either the wild-type or Rid1 form of gD. We found that cells resistant to HSV-1-induced cell fusion became susceptible when they expressed HveA and that the ability of HveA to mediate this fusion was dependent on the form of gD expressed.

MATERIALS AND METHODS

Virus and cells. Vero (African green monkey kidney) cells were obtained from the American Type Culture Collection and passaged in medium 199 with Hanks' salts (GibcoBRL) supplemented with 5% fetal bovine serum (Sigma). HEp-2 cells transfected with a plasmid expressing HSV-1 gD (H-gD-1) or vector control (H-control) (25) were passaged in Dulbecco's modification of Eagle's medium (GibcoBRL) supplemented with 10% fetal bovine serum and Geneticin (200 μg/ml; GibcoBRL). CHO cells constitutively expressing HveA (CHO-HVEM12), carrying a control plasmid (CHO-C8), or inducibly expressing lacZ (CHO-IEβ8) (36) were passaged in Ham's F12 medium (GibcoBRL) supplemented with 10% fetal bovine serum and either Geneticin (400 μg/ml, CHO-HVEM12 and CHO-C8) or puromycin (5 μg/ml, CHO-IEβ8; CloneTECH). CHO-IEβ8 cells stably expressing HveA were obtained by transfecting CHO-IEβ8 cells with pBEC10 (36), using DOSPER liposomal transfection reagent (Boehringer Mannheim) according to the manufacturer's directions and selecting for stable transfectants in Ham's F12 medium with 10% fetal calf serum containing puromycin at 150 μg/ml and Geneticin at 250 μg/ml. A clone expressing the desired phenotype (designated CHO-IEβ8/HveA) was maintained in the same medium. CHO-IEβ8 and CHO-IEβ8/HveA both inducibly express β-galactosidase after infection by HSV strains carrying the wild-type transactivator VP16, while CHO-HVEM12 and CHO-IEβ8/HveA both constitutively express HveA.

The following viruses were used: HSV-1(KOS) (abbreviated here KOS) and HSV-1(KOS)804 (KOS-804) (both provided by P. Schaffer, University of Pennsylvania) (28), HSV-1(KOS)Rid1 (KOS-Rid1) (9), HSV-1(MP) (22), and HSV-1(KOS)SR1 (KOS-SR1). Virus strains were passaged at low multiplicity in HEp-2 cells, except KOS-Rid1, which was passaged at low multiplicity in H-gD-1 cells under selective conditions.

The mutant KOS-SR1 is a Syn recombinant isolated from the coinfection of Vero cells with KOS-804 and KOS-Rid1 and carries the mutant gK and gD alleles of the parental strains. Vero cells were coinfected with 10 PFU each of KOS-804 and KOS-Rid1 per cell. After cytopathic effect was complete, lysates were prepared from infected cells by subjecting them to three freeze-thaw cycles followed by brief sonication. Titers of progeny virions were determined on Vero cells. Plaques developed in a liquid overlay of medium 199 supplemented with 1% heat-inactivated calf serum and 0.5% methylcellulose. Syncytial plaques were harvested and amplified on Vero cells. Aliquots of amplified plaque stocks were used in two ways, to infect H-gD-1 cells and to prepare viral DNA for use as template in amplification and restriction analysis of the gD gene (primers gD-5 and gD-7). Isolates with the ability to infect H-gD-1 cells (i.e., interference resistant), and shown by PCR to lack the *Pvu*II site found in the wild-type gD gene but missing in the KOS-Rid1 gD gene, were subjected to further rounds of plaque purification. Following three rounds of plaque purification, a recombinant was obtained and designated KOS-SR1.

DNA sequence analysis. Viral DNA was isolated and purified as previously described (33, 41). PCR was performed with Vent DNA polymerase (New England Biolabs), purified viral DNA, and primers specific for amplifying either the entire gK gene (gK-1 and gK-2 [41]), a portion of the gK gene spanning only the 804 gK Syn mutation (804SYN1 [5′-GAGCGTGTTCCTGCAGTACC-3′] and 804SYN2 [5′-CCGAGAGGATGATGGAACAG]), or a portion of the gD gene spanning the mutation responsible for the interference-resistant phenotype (gD-5 and gD-7 [9]). Products generated from primers gK-1 and gK-2 were used

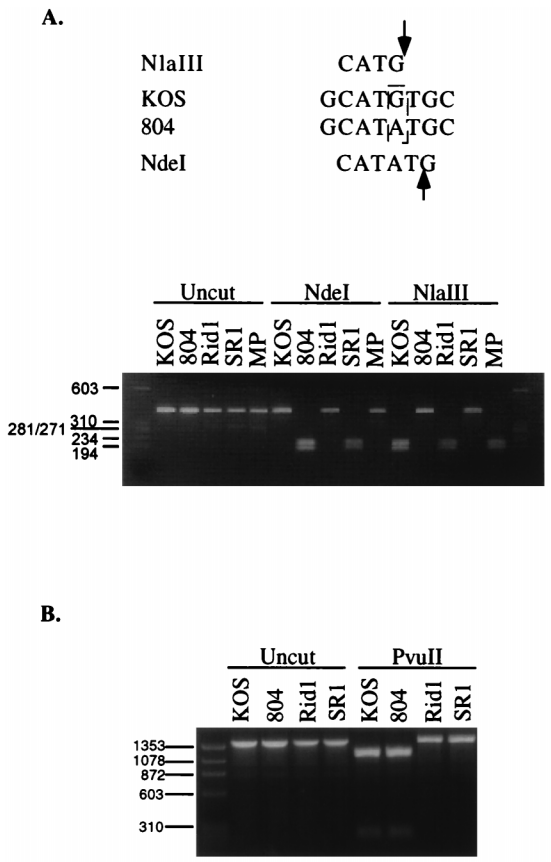

FIG. 1. Genotypic verification of mutations. (A) Characterization of the KOS-804 and KOS-SR1 gK Syn mutation. The G728A mutation in KOS-804 changes an *Nla*III site to an *Nde*I site. PCR-amplified fragments of the gK ORF were obtained from KOS, KOS-804, KOS-Rid1, KOS-SR1, and MP and then digested with *Nla*III or *Nde*I. (B) Characterization of the Rid1 and SR1 gD mutation. PCR-amplified fragments of the gD ORF were obtained from KOS, KOS-804, KOS-Rid1, and KOS-SR1 and digested with *Pvu*II, which cleaves only the wild-type gD gene. Sizes are indicated in base pairs.

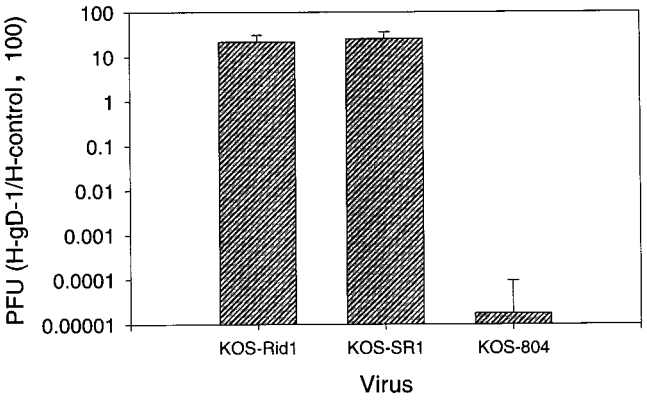

FIG. 2. Relative infectivities of KOS-804, KOS-Rid1, and KOS-SR1 for gD-expressing and control HEp-2 cells. Plaque assays were performed in duplicate on monolayers of H-gD-1 (gD-expressing) and H-control cells by infecting them with serial 10-fold dilutions of KOS-804, KOS-Rid1, and KOS-SR1. In each experiment, H-gD-1 and H-control cells were exposed in parallel to a single dilution series of each virus tested. Cultures having 50 to 500 plaques per flask were counted to determine the virus titer on each cell type. The titer of each virus stock as determined on gD-expressing cells was divided by the titer determined in parallel on H-control cells and then multiplied by 100 to yield the normalized data shown. The results presented are the mean values of these percentages obtained from two independent experiments, and the error bars represent the standard deviation.

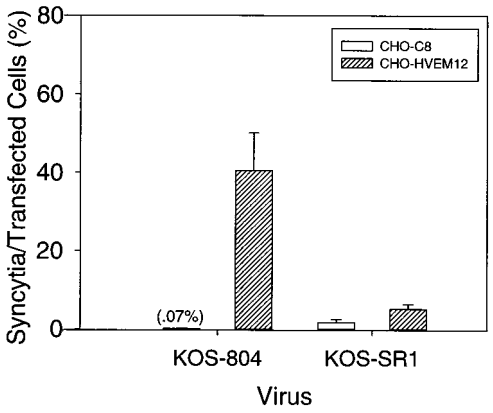

FIG. 4. HveA-mediated enhancement of cell fusion induced by KOS-804. HveA-expressing CHO-HVEM12 cells or control CHO-C8 cells were transfected with genomic DNA from KOS-804 or KOS-SR1. At 24 h posttransfection, cells were fixed and stained for gB to identify transfected cells. Frequency of syncytium induction is presented as syncytia with ≥10 nuclei/total number of transfected cells. Results shown are means and standard deviations from three separate experiments, each done in duplicate.

as template for sequencing, whereas products from primers 804SYN1 and 804SYN2 were used in marker transfer and marker repair experiments and in restriction analysis. PCR products obtained from primers gD-5 and gD-7 were also used in restriction analysis. Where indicated, PCR products were purified by using the Qiaex II gel extraction kit desalting protocol (Qiagen). Concentrations of purified PCR fragments were spectrophotometrically determined. Nucleotide sequencing was performed by both manual and automated protocols. Manual sequencing was performed with a Sequenase PCR product sequencing kit (United States Biochemical). Automated sequencing was performed with an ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and ABI 373 sequencer, in accordance with the manufacturer-recommended protocol. Long Ranger 6% (J. T. Baker Chemical Co.) and SeaQuate 6% (Sooner Scientific) polyacrylamide gels were used in the manual and automated protocols, respectively. Six primers were used to sequence the PCR-amplified gK gene. The three sense primers, gK-17 (5′-CGCCAAATGCGACAGCAACC-3′), gK-19 (5′ ATCGTCGGATCATGAAGC-3′), and gK-22 (5′ GTCATCGTAGG CTGCGAG-3′), and one antisense primer, gK-35 (5′-CCAGACGCACCCGTG TGTAC-3′), were previously described (11). The two remaining antisense primers used were gK-26A (5′-GCATCAACTCGCAGCCTACG-3′) and gK-36A (5′-CATATGCCGTTCCGGTTTCCGC-3′). Sequence data were analyzed by using the PCGENE program.

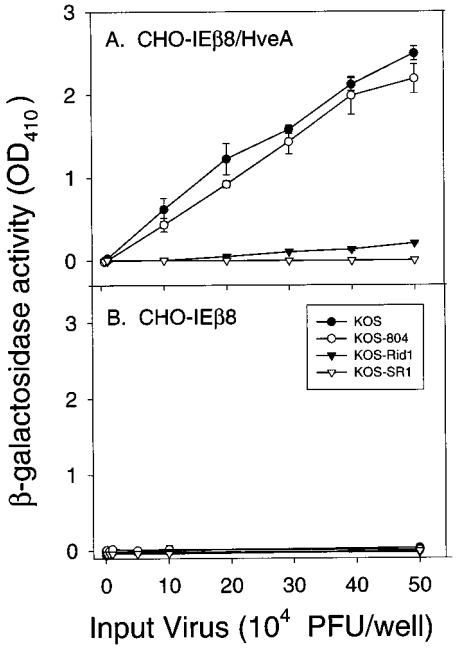

FIG. 3. Entry characteristics of KOS, KOS-804, KOS-Rid1, and KOS-SR1 on CHO-IEβ8 and CHO-IEβ8/HveA cells. Titers of KOS, KOS-804, KOS-Rid1, and KOS-SR1 were determined on 96-well plates of CHO-IEβ8 and CHO-IEβ8/HveA cells. Six hours postinfection, the plates were rinsed with PBS and solubilized in PBS–0.5% NP-40 supplemented with ONPG (Sigma) at 3 mg/ml. Plates were read in a Spectramax 250. Results shown are the mean and standard deviations of triplicate determinations. OD_{410}, optical density at 410 nm.

Plaque assays. Routine titrations of virus were performed by plaque assay on Vero cells, which were maintained after infection in medium 199 supplemented with 1% heat-inactivated calf serum (Sigma) and 0.1% human gamma globulin (Armour Pharmaceutical Co.). After 2 to 3 days of incubation, the cell monolayers were stained with Giemsa stain for the quantitation of plaques. Plaque assays were also performed with gD-expressing and control HEp-2 cells. Plaques were visualized by a modification of a previously described immunoassay (21), which was also used to detect syncytial foci in CHO-HVEM12 and CHO-C8 cells. Briefly infected cells were incubated sequentially with anti-gB monoclonal antibody II-105 (diluted 1:1,000) (40), biotin-conjugated goat anti-mouse immunoglobulin G (GibcoBRL), and streptavidin–β-galactosidase (GibcoBRL), followed by an overlay with ferricyanide buffer (41) supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 0.5 mg/ml; GibcoBRL). Plaques were visualized as blue foci.

Marker transfer and marker rescue. Fragments of the gK open reading frame (ORF) spanning the KOS-804 G728A mutation (using primers 804SYN1 and 804SYN2) were amplified from KOS-804 and KOS. Purified KOS or KOS-804 viral genomic DNA and purified PCR fragments were cotransfected into subconfluent Vero cell monolayers at a mass ratio of 1:5 (viral genomic DNA/PCR fragments), using the LipofectAMINE protocol (GibcoBRL). When cytopathic effect was complete (4 to 5 days posttransfection), lysates were prepared (three freeze-thaw cycles followed by brief sonication) and titers for quantification of recombinant progeny were determined on Vero cells.

Antibodies. Monoclonal antibodies to gB (II-105), gD (III-174), gH (52S), gL (VIII-200), and gC (VII-13-7) were previously described (38–40, 50). Monospecific antiserum (R140) against HveA was generated by immunizing a rabbit with baculovirus-produced HVEM(200t) (55). Purified protein (100 μg) mixed with an equal volume of complete Freund's adjuvant was injected subcutaneously and intramuscularly. The animal was boosted via injection by the same routes at 2-week intervals (total of four boosts) with 50 μg of protein mixed with an equal volume of incomplete Freund's adjuvant.

Fusion quantitation assays. Purified KOS-804 or KOS-SR1 viral DNA was transfected into CHO-HVEM12 and CHO-C8 cells by the LipofectAMINE protocol. Approximately 20 h posttransfection, the cells were fixed with methanol and transfected cells were identified by using the immunoassay detailed above. Blue foci were categorized according to the number of nuclei and counted. To quantitate the effects of anti-HSV antibodies on HveA-mediated fusion, CHO-IEβ8/HveA cells were infected with KOS-804 at 5 PFU/cell. Following a 2-h

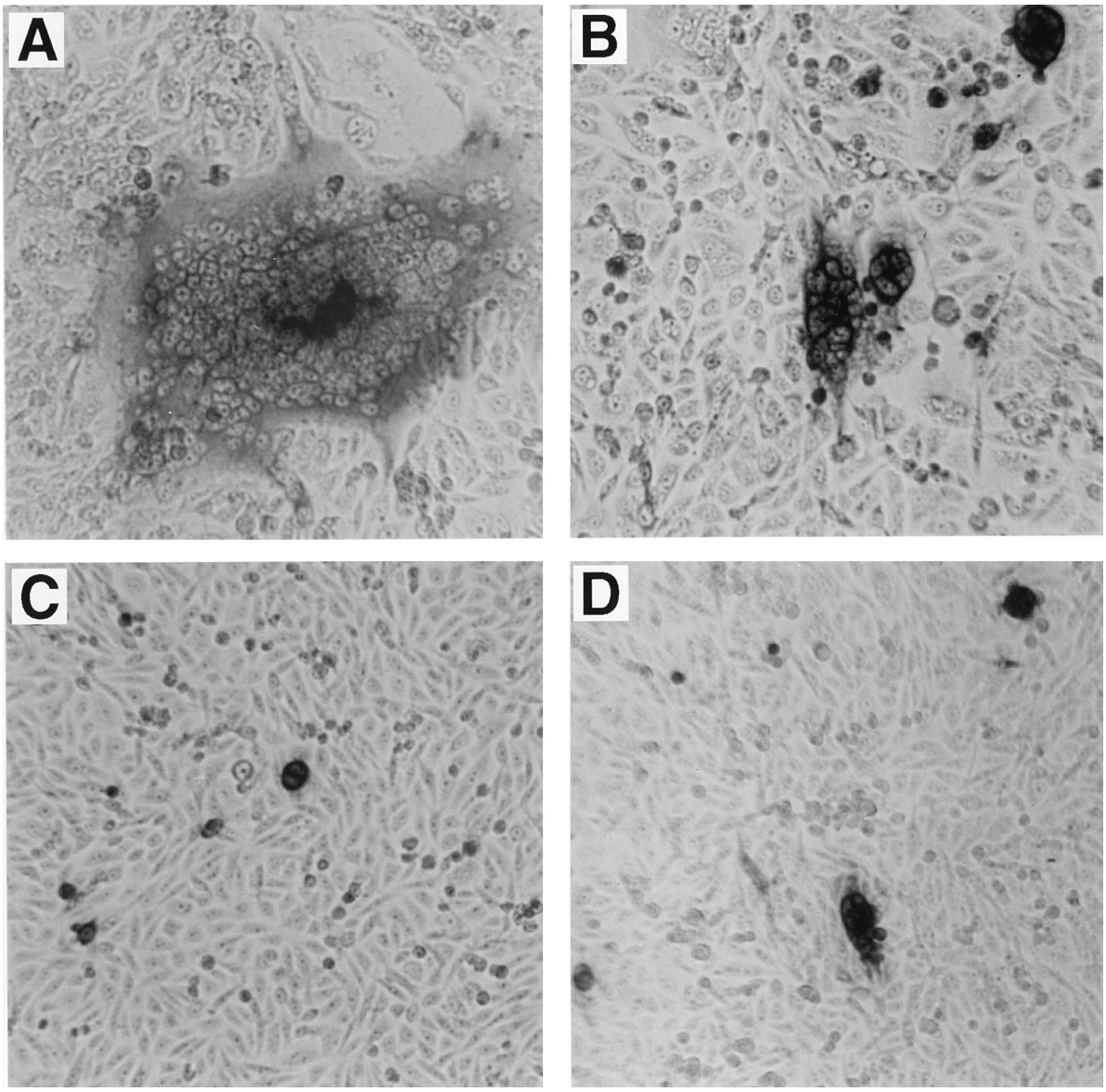

FIG. 5. Syncytia induced by KOS-804 or KOS-SR1 transfection of HveA-expressing and control CHO cells. CHO-HVEM12 and CHO-C8 cells were transfected with either KOS-804 or KOS-SR1 DNA and fixed 20 to 24 h later. Transfected cells were identified by staining for gB expression. (A and B) Transfection-induced syncytia and mononucleated cells representative of those found in CHO-HVEM12 cells transfected with KOS-804 and KOS-SR1, respectively; (C [KOS-804] and D [KOS-SR1]) results obtained by transfecting CHO-C8 cells.

adsorption period, the virus inoculum was removed and replaced with Ham's F12 medium supplemented with 2% heat-inactivated serum and various amounts of anti-HSV antibodies: anti-gD III-174 (38–40), anti-gH 52S (39, 50), anti-gL VIII-200 (39), and anti-gC VII-13-7 (39). Approximately 24 h postinfection, cells were fixed (0.5% glutaraldehyde in phosphate-buffered saline [PBS]), permeabilized (1 mM $MgCl_2$, 0.01% deoxycholic acid, 0.02% Nonidet P-40 [NP-40]), and overlaid with ferricyanide buffer supplemented with X-Gal (0.5 mg/ml).

For the cell-mixing assay, CHO-C8 cells were transfected with either KOS-804 or KOS-SR1 DNA. To minimize background β-galactosidase activity from nonfusogenic events (i.e., cell-cell spread of intact virus), both viral DNAs were digested with *Spe*I, which cuts the genome once in genes that do not influence cell fusion, and the digests were heat inactivated prior to transfection. Six hours posttransfection, the cells were detached by rinsing twice with Versene (PBS supplemented with 0.4% EDTA) followed by trypsin and reseeded with untransfected CHO-IEβ8/HveA cells. Cells were reseeded into both 96-well culture dishes (quantitative analysis) and 24-well culture dishes (qualitative analysis) in the absence or presence of various concentrations of anti-HveA serum or preimmune control serum control. Approximately 24 h postplating, the 24-well plates were fixed, permeabilized, and overlaid with ferricyanide buffer supplemented with X-Gal (0.5 mg/ml; GibcoBRL), while the 96-well plates were rinsed with PBS and solubilized in PBS–0.5% NP-40 supplemented with *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) at 3 mg/ml. Plates were read in a Spectramax 250.

RESULTS

Construction of KOS-SR1 and characterization of KOS-804 and KOS-SR1 mutations. Because the ability of HSV-1 to use HveA as a coreceptor for entry depends on the form of gD expressed, we tested two Syn strains of virus that expressed different forms of gD for the ability to induce the fusion of HveA-expressing cells. One strain was KOS-804, which is a Syn mutant isolated from mutagenized stocks of KOS (47); the other was KOS-SR1, a recombinant that was plaque purified from Vero cells coinfected with KOS-804 and KOS-Rid1. KOS-Rid1 is a previously characterized mutant whose mutation in its gD gene (Q27P) allows it to overcome gD-mediated

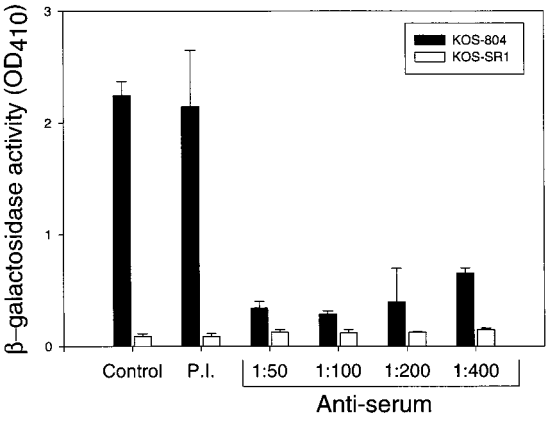

FIG. 6. Inhibition of KOS-804-induced syncytium formation by anti-HveA antibodies. CHO-C8 cells were transfected with heat-inactivated *Spe*I digests of either KOS-804 or KOS-SR1 DNA. Six hours posttransfection, cells were detached and reseeded into 96-well plates with similarly detached, untransfected CHO-IEβ8/HveA cells. Anti-HveA or preimmune serum was included in the medium at a dilution range of 1:50 to 1:400. Approximately 20 h postreseeding, the cells were rinsed with PBS and solubilized in PBS–0.5% NP-40 supplemented with ONPG (Sigma) at 3 mg/ml. Plates were read in a Spectramax 250. Results shown are the mean and standard deviations of triplicate determinations. Control, no serum added; P.I., preimmune at 1:50 dilution; OD_{410}, optical density at 410 nm.

interference (9) while significantly reducing its ability to use HveA for entry (36).

KOS-804 forms syncytial plaques on Vero cells and nonsyncytial plaques on HEp-2 cells (28). Though its Syn mutation, initially mapped to the UL1 locus (28), was later accurately mapped to the gK gene (44), the exact nature of the Syn mutation remained unknown. Sequencing of the KOS-804 gK gene (GenBank accession no. AF035012) revealed two nucleotide substitutions (C360T and G728A) in comparison to the KOS gK gene. The C360T substitution is silent whereas the G728A substitution results in a C243Y amino acid substitution. Confirmation that the C243Y amino acid substitution was responsible for the KOS-804 Syn phenotype was obtained through marker transfer and marker rescue experiments. PCR-amplified DNA fragments of the gK ORF from KOS-804 or KOS were cotransfected with KOS or KOS-804 DNA, respectively. The gK DNA fragment from KOS-804 transferred the Syn phenotype to KOS, while the corresponding fragment from KOS rescued the Syn mutation of KOS-804. Thus, fragments spanning the C243Y amino acid substitution (amino acids 172 to 305) could transfer or rescue the Syn phenotype, depending on the DNA template used for PCR and the recipient genome (Table 1).

Verification of the nucleotide substitutions responsible for the gD and gK mutations by PCR amplification and restriction endonuclease analysis are illustrated in Fig. 1. The KOS-804 gK Syn mutation G728A changes an *Nla*III site to a unique *Nde*I site in the gK gene (Fig. 1A), while the KOS-Rid1 gD mutation is characterized by the loss of a *Pvu*II site (Fig. 1B). KOS-SR1 displayed the mutant gD and gK genotypes of both KOS-Rid1 and KOS-804, respectively. KOS-SR1 also displayed the biological phenotypes of both of its mutant parents: it is syncytial in Vero cells and nonsyncytial in HEp-2 cells like its parent KOS-804 (data not shown) and is resistant to gD-mediated interference like its parent KOS-Rid1 (9) (Fig. 2).

We then examined the ability of KOS-SR1 to infect HveA-expressing CHO cells in comparison to its parents, KOS-804 and KOS-Rid1, as well as wild-type KOS. We found that viruses expressing the wild-type form of gD (KOS and KOS-804) were able to infect the HveA-expressing CHO cells, whereas viruses expressing the KOS-Rid1 form of gD (KOS-Rid1 and KOS-SR1) were greatly impaired in the ability to use HveA for entry (Fig. 3). Consistent with previous findings (36), the ability of these viruses to infect HveA-expressing CHO cells was determined by the form of gD expressed and not on the presence or absence of the gK Syn mutation.

Effect of HveA expression on susceptibility of CHO cells to cell fusion induced by KOS-804 and KOS-SR1. CHO cells expressing HveA are susceptible to KOS and KOS-804 infection, whereas control CHO cells (transfected with empty vector) are resistant (36). Both cell types can be transfected with genomic viral DNAs and can support HSV gene expression with equivalent efficiencies. Therefore, it was necessary to introduce viral genomes into HveA-expressing (CHO-HVEM12) and control (CHO-C8) cells by transfection in order to assess the effects of HveA expression on HSV-induced cell fusion. Figure 4 summarizes the results of experiments in which CHO-HVEM12 and control CHO-C8 cells were transfected with either KOS-SR1 or KOS-804 DNA. Transfected cells expressing gB were counted and scored as to the number of nuclei per cell. Approximately 40% of CHO-HVEM12 cells transfected with KOS-804 DNA formed large syncytia (>10 nuclei/cell), compared with fewer than 1% of transfected control cells. These results show that HveA can render cells susceptible to HSV-1-induced cell fusion. In contrast, 5.1% of CHO-HVEM12 cells transfected with KOS-SR1 DNA formed large syncytia (>10 nuclei/cell), compared with 40% of cells transfected with KOS-804 DNA, indicating that the form of gD expressed by the transfected virus influenced the efficiency of syncytium formation on HveA-expressing cells. Although very few large syncytia were observed on the control cells transfected with either virus, KOS-SR1 DNA clearly induced cell fusion more efficiently on control cells than did KOS-804 DNA. This finding is consistent with previous findings that the Rid1 mutation slightly enhanced the ability of KOS-Rid1, in comparison with KOS, to infect control CHO cells (9). Representative photographs of syncytia formed by KOS-SR1 and KOS-804 on both cell lines are shown in Fig. 5.

Effects of anti-HveA antibodies on cell fusion induced by KOS-804 and KOS-SR1 on HveA-expressing cells. To determine the effect of anti-HveA antibodies on HveA-mediated fusion, we used a cell-mixing assay. CHO-C8 cells were transfected with heat-inactivated *Spe*I digests of either KOS-804 or KOS-SR1 DNA. *Spe*I does not cleave genes essential for fusion induction but minimizes the production of viable progeny virions. Six hours posttransfection, the cells were reseeded with untransfected CHO-IEβ8/HveA cells, in the absence or presence of various concentrations of anti-HveA serum or preimmune control serum. Because CHO-IEβ8/HveA cells constitutively express HveA and inducibly express β-galactosidase after HSV-1 infection (due to transactivating activity of the virion tegument protein VP16), blue syncytial foci would result only when transfected CHO-C8 cells fused with CHO-IEβ8/HveA cells. Approximately 24 h postreseeding, the cells were analyzed for β-galactosidase activity. Figure 6 shows that while the anti-HveA serum was a potent inhibitor of fusion mediated by KOS-804, it had no effect on the fusion mediated by KOS-SR1. Figure 7 shows that the anti-HveA serum also reduced the size of syncytia induced by KOS-804, whereas no such effect was observed for syncytia induced by KOS-SR1. These results illustrate that the low levels of cell fusion induced by KOS-SR1 were largely independent of HveA expression, whereas cell fusion induced by KOS-804 was heavily HveA

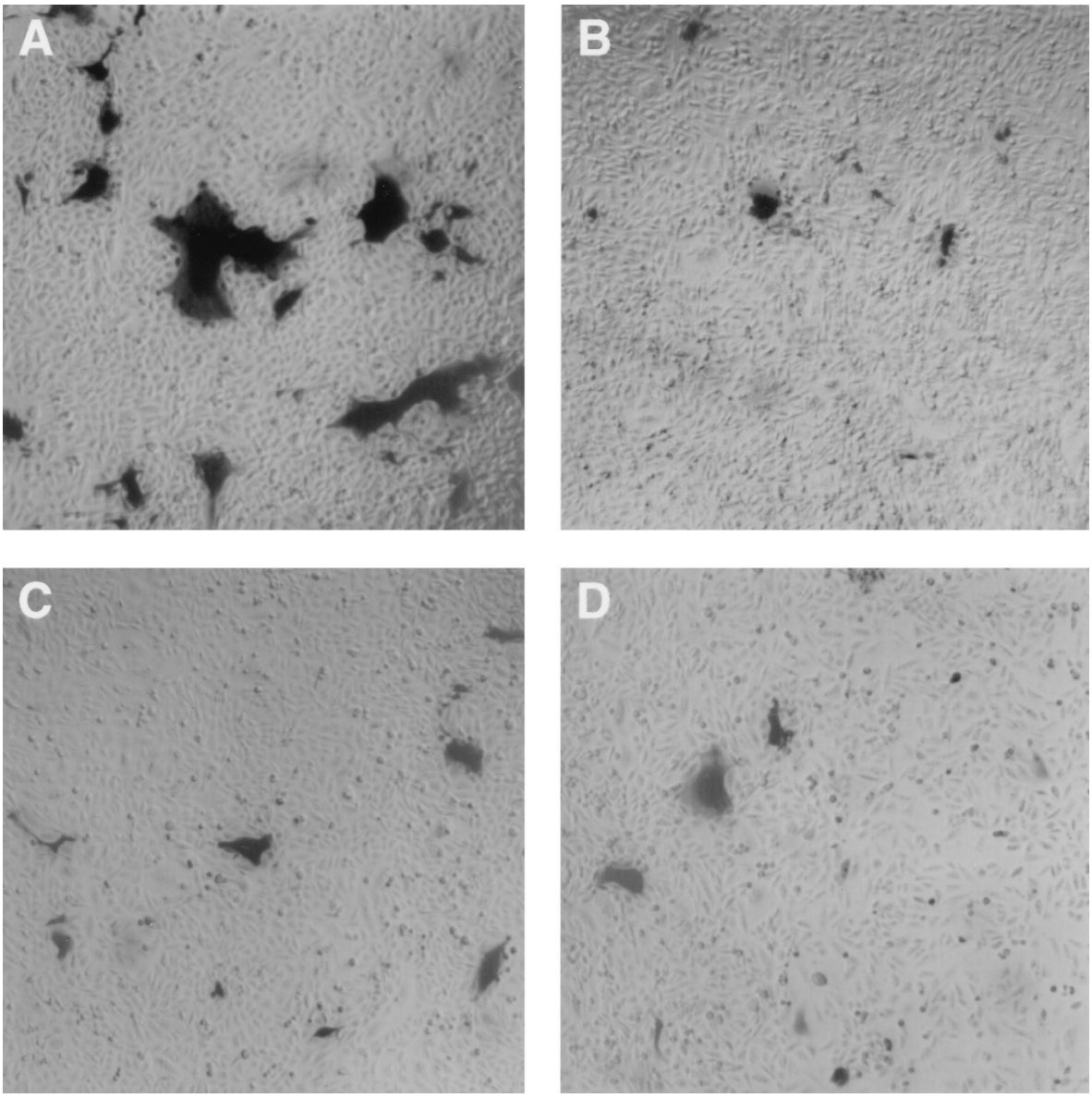

FIG. 7. Reduction in sizes of KOS-804 Syn foci by anti-HveA antibodies. The assay was performed as described for Fig. 6 except that mixed populations of cells were reseeded into 24-well plates in the absence or presence of various concentrations of anti-HveA serum. (A and B) Typical Syn plaques formed by KOS-804 and KOS-SR1, respectively, in the presence of preimmune serum at a dilution of 1:50; (C and D) typical Syn plaques formed by KOS-804 (dilution of 1:100) and KOS-SR1 (dilution of 1:50), respectively, in the presence of anti-HveA serum.

dependent. Inhibitory effects of the anti-HveA antibodies were observed at serum dilutions of up to 1:400.

Effects of anti-HSV antibodies on cell fusion induced by KOS-804 on HveA-expressing cells. Monoclonal antibodies to viral glycoproteins gD, gH, gL, and gC were evaluated for the ability to inhibit HSV-1-induced fusion of HveA-expressing cells. CHO-IEβ8/HveA cells were infected with KOS-804 at 5 PFU/cell. Two hours postinfection, antibodies to the above-mentioned glycoproteins were added at various concentrations. The extent of cell fusion was determined 20 h postinfection, when the cells were fixed and stained for β-galactosidase expression. The results presented in Table 2 and Fig. 8 show that the gD, gH, and gL antibodies all provided some fusion inhibition. The anti-gD antibody completely blocked fusion with the result that cytopathic effects characteristic of nonsyncytial HSV strains (cell rounding) were observed instead of fusion. The anti-gH antibody was almost as effective as the anti-gD antibody in blocking cell fusion. The anti-gL antibody was much less effective in contrast to its potent activity in inhibiting KOS-804-induced fusion of Vero cells (39). The anti-gC antibody, as expected, had little effect.

DISCUSSION

Cellular factors previously shown to function in entry and cell fusion include GAGs such as heparan sulfate (48, 49). Besides GAGs, other cellular determinants of susceptibility to HSV-induced cell fusion were largely unknown. In this study, we focused on HveA, a recently identified cellular mediator of HSV-1 entry, as a possible coreceptor of HSV-induced cell

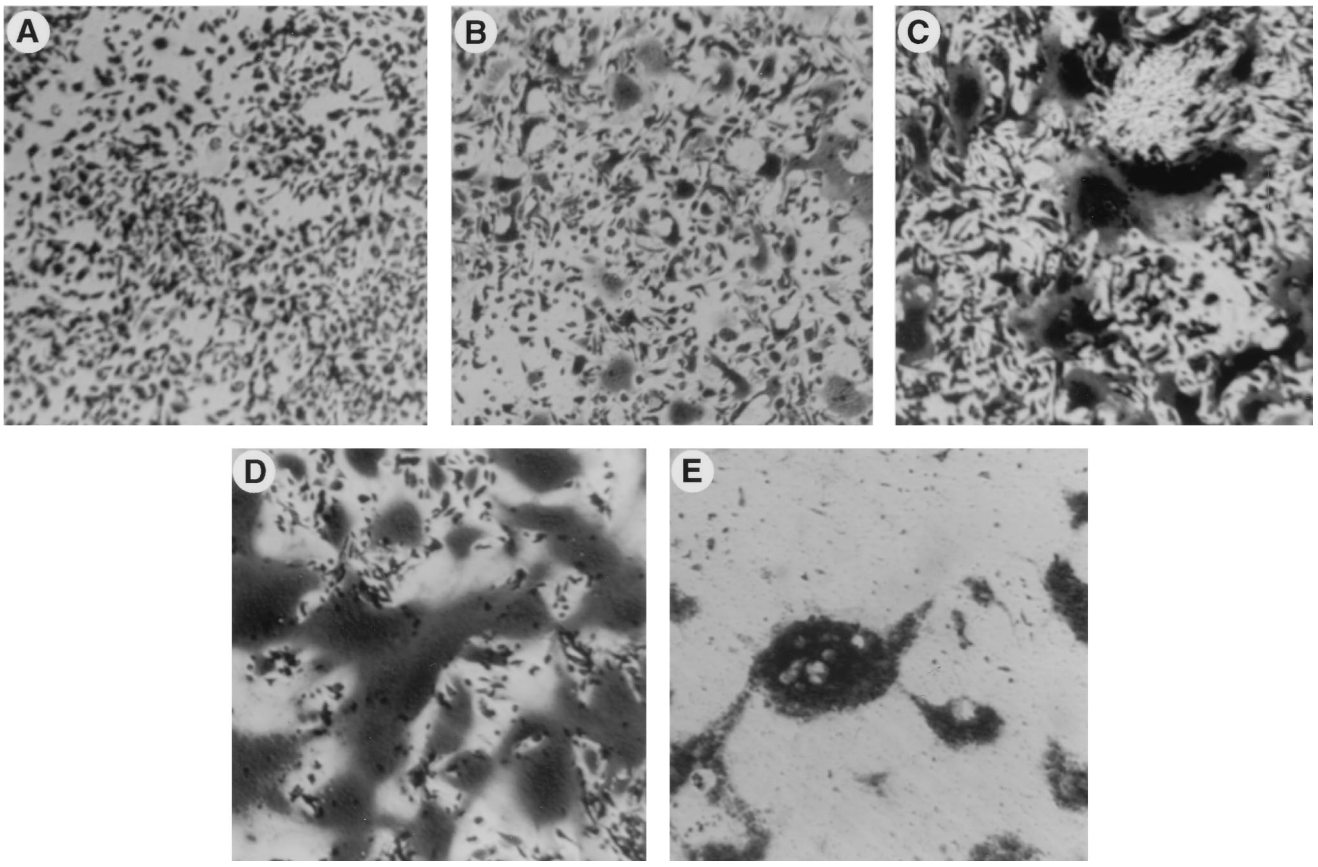

FIG. 8. Inhibition of HveA-mediated cell fusion by anti-HSV monoclonal antibodies. CHO-IEβ-8/HveA cells were infected with KOS-804 at 5 PFU/cell. The cells were overlaid with medium containing anti-gD (III-174) at 350 μg/ml (A), anti-gH (52S) at 500 μg/ml (B), anti-gL (VIII-200) at 350 μg/ml (C), anti-gC (VII-13-7) at 500 μg/ml (D), or no antibody (E). Approximately 20 h postinfection, cells were fixed, exposed to β-galactosidase substrate, and photographed.

fusion. We found that in cell lines normally resistant to both viral entry and cell fusion induced by Syn mutants of HSV-1(KOS), HveA expression significantly increased their susceptibility to fusion induced by a KOS Syn mutant expressing the wild-type (KOS-804), but not the Rid1 (KOS-SR1), form of gD (Fig. 4 and 5). Moreover, anti-HveA antibodies inhibited the cell fusion induced by KOS-804. Thus, HveA can serve as a coreceptor for HSV-1-induced cell-cell fusion as well as viral entry, provided that viral gD is not altered by mutations found in Rid-1 gD.

It should be noted that GAG-positive wild-type CHO cells are partially susceptible to entry and fusion induced by HSV-1(MP) (48), another Syn variant, presumably due to its ability to utilize some hamster cell surface protein as a coreceptor. Interestingly, presence of the Rid1 gD mutation in KOS and KOS-804 enhances the ability of the viruses to infect (9) and induce fusion of control CHO cells, respectively, suggesting that the alteration in gD may allow limited usage of hamster coreceptors while reducing usage of HveA as coreceptor. This postulate is supported by the observation that anti-HveA antibodies did not inhibit KOS-SR1-induced fusion on HveA-expressing cells.

TABLE 2. Effects of antiglycoprotein antibodies on HveA-mediated fusion[a]

Antibody concn (μg/ml)	Inhibition of cell fusion[b]			
	gD (III-174)	gH (52S)	gL (VIII-200)	gC (VII-13-7)
50	++	++	++	−
150	+++	++	++	−
350	+++++	+++	++	−
500	+++++	++++	++	−

[a] The infected cells were examined microscopically to estimate the extent to which the antibodies inhibited cell fusion.

[b] In the absence of antibody (indicated in parentheses), essentially all nuclei were present in syncytia. In the presence of antibody, inhibition of fusion was estimated by the fraction of nuclei in single cells (−, less than 20%; +, 20%; ++, 40%; +++, 60%; ++++, 80%; +++++, 100%).

Though the syncytial mutations mapping to the gK gene are numerous (11), most alter codon 40 of the gene (11, 41). Sequencing of the KOS-804 gK gene revealed a previously unreported Syn mutation. Verification that this mutation (G728A) is responsible for the KOS-804 phenotype was provided by marker transfer and marker rescue experiments. Based on the orientation of gK in membranes proposed by Mo and Holland (35), the KOS-804 Syn mutation is located in the ectodomain of the protein, as are the other known gK Syn mutations (11). The role of gK in cell fusion is unknown, and it is unclear how the KOS-804 amino acid substitution (C243Y) or any of the many Syn mutations that map to gK affect the normal function of the protein.

Antibodies that can block cell fusion include several specific for gB, gD, gH, and gL (16, 34, 37–40). In this study, the concentrations of antiglycoprotein antibodies required to completely inhibit fusion (Table 2) were higher than those required

to inhibit fusion in other cell lines such as Vero cells, particularly the anti-gL antibodies (39). That the gD antibodies were most effective at inhibiting HveA-mediated fusion is consistent with the finding that gD interacts directly with HveA (55). The inability of anti-gH and anti-gL antibodies to provide complete inhibition of fusion is somewhat puzzling, as these antibodies very effectively inhibited fusion in Vero cells (39). A possible explanation is that the precise roles of the viral glycoproteins in inducing cell fusion, and the epitopes available for binding to inhibitory antibodies, may depend on the particular coreceptor with which these glycoproteins interact.

Viruses expressing the Rid1 form of gD have greatly reduced ability to infect HveA-expressing CHO cells (36) yet are competent to infect a variety of human and animal cell types, suggesting that they must utilize other cellular receptors for entry and, where applicable, for fusion as well.

ACKNOWLEDGMENTS

We thank N. Susmarski and M. L. Parish for excellent technical assistance.

This work was supported by grants from the NIH to P.G.S. (R01 CA21776) and to G.H.C. and R.J.E. (PO1 NS30606, RO1 NS36731, and RO1 AI18239). T.T.-A. was supported by National Research Service Award F31 GM15056.

REFERENCES

1. **Baines, J. D., P. L. Ward, G. Campadelli-Fiume, and B. Roizman.** 1991. The U_L20 gene of herpes simplex virus 1 encodes a function necessary for viral egress. J. Virol. **65:**6414–6424.
2. **Balan, P., N. Davis-Poynter, S. Bell, H. Atkinson, H. Browne, and T. Minson.** 1994. An analysis of the *in vitro* and *in vivo* phenotypes of mutants of herpes simplex type 1 lacking glycoproteins gG, gE, gI or the putative gJ. J. Gen. Virol. **75:**1245–1258.
3. **Bond, V. C., and S. Person.** 1984. Fine structure physical map locations of alterations that affect cell fusion in herpes simplex virus type 1. Virology **132:**368–376.
4. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137:**185–190.
5. **Bzik, D. J., and S. Person.** 1981. Dependence of herpes simplex virus type 1-induced cell fusion on cell type. Virology **110:**35–42.
6. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
7. **Chatterjee, S., J. Koga, and R. J. Whitley.** 1989. A role for herpes simplex virus type 1 glycoprotein E in induction of cell fusion. J. Gen. Virol. **70:** 2157–2162.
8. **Davis-Poynter, N., S. Bell, T. Minson, and H. Browne.** 1994. Analysis of the contributions of herpes simplex virus type 1 membrane proteins to the induction of cell-cell fusion. J. Virol. **68:**7586–7590.
9. **Dean, H. J., S. S. Terhune, M. T. Shieh, N. Susmarski, and P. G. Spear.** 1994. Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity. Virology **199:**67–80.
10. **DeLuca, N., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology **122:**411–423.
11. **Dolter, K. E., R. Ramaswamy, and T. C. Holland.** 1994. Syncytial mutations in the herpes simplex virus type 1 gK (UL53) gene occur in two distinct domains. J. Virol. **68:**8277–8281.
12. **Ejercito, P. M., E. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2:**357–364.
13. **Engel, J. P., E. P. Boyer, and J. L. Goodman.** 1993. Two novel single amino acid syncytial mutations in the carboxy terminus of glycoprotein B of herpes simplex virus type 1 confer a unique pathogenic phenotype. Virology **192:** 112–120.
14. **Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson.** 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J. Virol. **66:**341–348.
15. **Gage, P. J., M. Levine, and J. C. Glorioso.** 1993. Syncytium-inducing mutations localize to two discrete regions within the cytoplasmic domain of herpes simplex virus type 1 glycoprotein B. J. Virol. **67:**2191–2201.
16. **Gompels, U., and A. Minson.** 1986. The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology **153:**230–247.
17. **Haanes, E. J., C. M. Nelson, C. L. Soule, and J. L. Goodman.** 1994. The UL45 gene product is required for herpes simplex virus type 1 glycoprotein B-induced fusion. J. Virol. **68:**5825–5834.
18. **Herold, B. C., R. J. Visalli, N. Susmarski, C. R. Brandt, and P. G. Spear.** 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein B. J. Gen. Virol. **75:**1211–1122.
19. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J. Virol. **65:**1090–1098.
20. **Hoggan, M. D., and B. Roizman.** 1959. The isolation and properties of a variant of *herpes simplex* producing multinucleated giant cells in monolayer cultures in the presence of antibody. Am. J. Hyg. **70:**208–219.
21. **Holland, T. C., R. M. Sandri-Goldin, L. E. Holland, S. D. Marlin, M. Levine, and J. C. Glorioso.** 1983. Physical mapping of the mutation in an antigenic variant of herpes simplex virus type 1 by use of an immunoreactive plaque assay. J. Virol. **46:**649–652.
22. **Hutchinson, L., H. Browne, V. Wargent, N. Davis-Poynter, S. Primorac, K. Goldsmith, A. C. Minson, and D. C. Johnson.** 1992. A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH. J. Virol. **66:**2240–2250.
23. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66:**5603–5609.
24. **Jacobson, J. G., S. L. Martin, and D. M. Coen.** 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. J. Virol. **63:**1839–1843.
25. **Johnson, R. M., and P. G. Spear.** 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. J. Virol. **63:**819–827.
26. **Lee, G. T.-Y., and P. G. Spear.** 1980. Viral and cellular factors that influence cell fusion induced by herpes simplex virus. Virology **107:**402–414.
27. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62:**1486–1494.
28. **Little, S. P., and P. A. Schaffer.** 1981. Expression of the syncytial (*syn*) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two *syn* loci. Virology **112:**686–702.
29. **Longnecker, R., and B. Roizman.** 1987. Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science **236:** 573–576.
30. **MacLean, C. A., L. M. Robertson, and F. E. Jamieson.** 1993. Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo. J. Gen. Virol. **74:**975–983.
31. **Manservigi, R., P. G. Spear, and A. Buchan.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74:**3913–3917.
32. **McKenzie, R. C., R. M. Epand, and D. C. Johnson.** 1987. Cyclosporine A inhibits herpes simplex virus-induced cell fusion but not virus penetration into cells. Virology **159:**1–9.
33. **Mettenleiter, T. C., and I. Rauh.** 1990. A glycoprotein gX-β-galactosidase fusion gene as insertional marker for rapid identification of pseudorabies viral mutants. J. Virol. Methods **30:**55–66.
34. **Minson, A. C., T. C. Hodgman, P. Digard, D. C. Hancock, S. E. Bell, and E. A. Buckmaster.** 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. J. Gen. Virol. **67:**1001–1013.
35. **Mo, C., and T. C. Holland.** 1997. Determination of the transmembrane topology of herpes simplex virus type 1 glycoprotein K. J. Biol. Chem. **272:** 33305–33311.
36. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427–436.
37. **Navarro, D., P. Paz, and L. Pereira.** 1992. Domains of herpes simplex virus I glycoprotein B that function in virus penetration, cell-to-cell spread, and cell fusion. Virology **186:**99–112.
38. **Noble, A. G., G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear.** 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology **129:**218–224.
39. **Novotny, M. J., M. L. Parish, and P. G. Spear.** 1996. Variability of herpes simplex virus 1 gL and anti-gL antibodies that inhibit cell fusion but not viral infectivity. Virology **221:**1–13.
40. **Para, M. F., M. L. Parish, A. G. Noble, and P. G. Spear.** 1985. Potent neutralizing activity associated with anti-glycoprotein D specificity among monoclonal antibodies selected for binding to herpes simplex virions. J. Virol. **55:**483–488.
41. **Pertel, P. E., and P. G. Spear.** 1996. Modified entry and syncytium formation by herpes simplex virus type 1 mutants selected for resistance to heparin inhibition. Virology **226:**22–33.
42. **Pogue-Geile, K. L., and P. G. Spear.** 1987. The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP. Virology **157:**67–74.

43. **Roller, R. J., and D. Rauch.** 1998. Herpesvirus entry mediator HVEM mediates cell-cell spread in BHK(TK$^-$) cell clones. J. Virol. **72:**1411–1417.
44. **Roop, C., L. Hutchinson, and D. C. Johnson.** 1993. A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. J. Virol. **67:**2285–2297.
45. **Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman.** 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. J. Virol. **29:**677–697.
46. **Sanders, P. G., N. M. Wilkie, and A. J. Davison.** 1982. Thymidine kinase deletion mutants of herpes simplex virus type 1. J. Gen. Virol. **63:**277–295.
47. **Schaffer, P. A., V. Vonka, R. Lewis, and M. Benyesh-Melnick.** 1970. Temperature-sensitive mutants of herpes simplex virus. Virology **70:**1144–1146.
48. **Shieh, M.-T., and P. G. Spear.** 1994. Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. J. Virol. **68:** 1224–1228.
49. **Shieh, M.-T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear.** 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J. Cell Biol. **116:**1273–1281.
50. **Showalter, S. D., M. Zweig, and B. Hampar.** 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP4. Infect. Immun. **34:**684–692.
51. **Spear, P. G.** 1993. Membrane fusion induced by herpes simplex virus, p. 201–232. *In* J. Bentz (ed.), Viral fusion mechanisms. CRC Press, Inc., Boca Raton, Fla.
52. **Spear, P. G.** 1987. Virus-induced cell fusion, p. 3–32. *In* A. E. Sowers (ed.), Cell fusion. Plenum Press, New York, N.Y.
53. **Visalli, R. J., and C. R. Brandt.** 1991. The HSV-1 UL45 gene product is not required for growth in Vero cells. Virology **185:**419–423.
54. **Walev, I., M. Lingen, M. Lazzaro, K. Weise, and D. Falke.** 1994. Cyclosporin A resistance of herpes simplex virus-induced "fusion from within" as a phenotypical marker of mutations in the syn 3 locus of the glycoprotein B gene. Virus Genes **8:**83–86.
55. **Whitbeck, J. C., C. Peng, H. Lou, R. Xu, S. H. Willis, M. Ponce de Leon, T. Peng, A. V. Nicola, R. I. Montgomery, M. S. Warner, A. Soulika, L. Spruce, W. T. Moore, J. D. Lambris, P. G. Spear, G. H. Cohen, and R. J. Eisenberg.** 1997. Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry. J. Virol. **71:**6083–6093.
56. **Wittels, M., and P. G. Spear.** 1991. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. Virus Res. **18:**271–290.
57. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. **63:**52–58.