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A Cell Surface Protein with Herpesvirus Entry Activity (HveB) Confers Susceptibility to Infection by Mutants of Herpes Simplex Virus Type 1, Herpes Simplex Virus Type 2, and Pseudorabies Virus

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Certain mutant strains of herpes simplex virus type 1 (HSV-1) are unable to infect cells in which entry is dependent on HVEM, the previously described herpesvirus entry mediator designated here as herpesvirus entry protein A (HveA). These mutant viruses can infect other cells where entry is apparently dependent on other co-receptors. The mutant virus HSV-1(KOS)Rid1 was used to screen a human cDNA expression library for ability of transfected plasmids to convert resistant Chinese hamster ovary cells to susceptibility to virus entry. A plasmid expressing the previously described poliovirus receptor-related protein 2 (Prr2) was isolated on the basis of this activity. This protein, designated here as HveB, was shown to mediate the entry of three mutant HSV-1 strains that cannot use HVEM as co-receptor, but not wild-type HSV-1 strains. HveB also mediated the entry of HSV-2 and pseudorabies virus but not bovine herpesvirus type 1. HveB was expressed in some human neuronal cell lines, fibroblastic cells, keratinocytes, and primary activated T lymphocytes. Antibodies specific for HveB blocked infection of HveB-expressing CHO cells and a human fibroblastic cell strain HEL299. Differences in ability of HSV-1 and HSV-2 strains to use HveB for entry should influence the types of cells that can be infected and thereby account in part for serotype and strain differences in tissue tropism and pathogenicity.

INTRODUCTION

Members of the alphaherpesvirus subfamily of the herpesviruses typically have a broad host range and a short replicative cycle, are highly cytotoxic to cultured cells, and can establish latent infections in cells of the nervous system of the natural host (Roizman, 1993). Human and animal representatives of the alphaherpesvirus subfamily exhibit common requirements for entry into cells (Mettenleiter, 1995; Spear, 1993). In most cases, five viral envelope glycoproteins (gB, gC, gD, gH, and gL) mediate virus binding to cells and entry. The initial interaction of virus with cells is the binding of gC, and in some cases also gB, to cell surface glycosaminoglycans (GAGs), preferentially heparan sulfate. Although gC is dispensible for the infection of many cultured cells, gB, gD, gH, and gL are required for mediating the fusion between virion envelope and cell membrane that allows viral penetration. Various lines of evidence point to interaction of some of these envelope glycoproteins, particularly gD, with cell surface receptors other than heparan sulfate and to competition among alphaherpesviruses for binding to gD receptors (Campadelli-Fiume *et al.,* 1988; Chase *et al.,* 1993; Johnson *et al.,* 1990; Johnson and Ligas, 1988; Karger and Mettenleiter, 1993; Lee and Fuller, 1993; Liang *et al.,* 1991; Petrovskis *et al.,* 1988).

Recently, an expression cloning assay was devised for isolating plasmids encoding cell surface proteins that can mediate herpes simplex virus type 1 (HSV-1) entry (Montgomery *et al.,* 1996). This assay relies on the use of Chinese hamster ovary (CHO) cells, which express GAGs required for virus binding to cells but are resistant to the entry of certain HSV-1 strains such as HSV-1(KOS) (Shieh *et al.,* 1992). Expression libraries, or subdivisions of the libraries, that contain plasmids capable of conferring susceptibility to HSV-1(KOS) can be identified by transfecting the CHO cells and then assaying for infection with a recombinant HSV-1(KOS) expressing a reporter gene.

The first cell surface protein shown by this assay to have herpesvirus entry activity is a previously undescribed member of the human TNF receptor family, which was originally named herpesvirus entry mediator (HVEM) and is here designated herpesvirus entry protein A (HveA) for reasons to be discussed below. HveA is a type I membrane glycoprotein with cysteine-rich repeats in the ectodomain that are characteristic of the TNF receptor family (Montgomery *et al.,* 1996) and with a cytoplasmic domain that can interact with members of

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the TRAF family of signalling molecules (Hsu *et al.,* 1997; Marsters *et al.,* 1997). HveA is a receptor for two members of the TNF family, lymphotoxin α and LIGHT (Mauri *et al.,* 1998). HveA also binds to isolated HSV-1 or HSV-2 gD and to gD in virions (Nicola *et al.,* 1998; Whitbeck *et al.,* 1997).

HveA is expressed in many fetal and adult human tissues, including lung, liver, and kidney (Montgomery *et al.,* 1996), but appears to be most abundantly expressed in lymphoid organs and cells (Hsu *et al.,* 1997; Kwon *et al.,* 1997; Marsters *et al.,* 1997). By use of anti-HveA antibodies that blocked HSV entry, it was shown that HveA serves as the principal co-receptor for entry of HSV-1(KOS) into activated human T lymphocytes (Montgomery *et al.,* 1996). The antibodies did not protect a number of other human cell types from infection, however, indicating that there must be other co-receptors for HSV entry.

Another indication for the existence of multiple independent co-receptors for HSV entry was the finding that, although HveA expression in CHO cells enhanced the entry of all wild-type HSV-1 and HSV-2 strains tested, HveA failed to mediate the entry of three mutant HSV-1 strains (Montgomery *et al.,* 1996). Two of these strains, designated HSV-1(KOS)Rid1 and HSV-1(KOS)Rid2 and abbreviated here KOS-Rid1 and KOS-Rid2, are viable mutants selected for their ability to overcome interference with viral entry imposed by the expression of wild-type HSV-1 gD in cells (Dean *et al.,* 1994). The third strain, HSV-1(ANG), was isolated from a clinical specimen (Munk and Donner, 1963) under conditions that would be expected to inhibit the replication of wild-type HSV (under an overlay of agar containing inhibitory sulfated polysaccharides) and perhaps select for viral variants. This strain proved to be as resistant to gD-mediated interference as KOS-Rid1 and KOS-Rid2 (Dean *et al.,* 1994). All three strains have amino acid substitutions, in gD at position 27 (Q27P or Q27R), that are sufficient to confer the mutant phenotype of resistance to gD-mediated interference. Consistent with failure of the mutant strains to use HveA for entry, the mutant forms of gD failed to bind HveA, whereas wild-type forms of HSV-1 and HSV-2 gD were able to bind (Whitbeck *et al.,* 1997). Because the mutant HSV-1 strains can infect a number of human or other cell types, despite failure to use HveA for entry, other co-receptors for entry must be expressed in these cells.

The aims of the studies described here were to isolate a human co-receptor that is functional for entry of KOS-Rid1 into resistant CHO cells and to explore the types of cells and herpesvirus strains for which entry via the new co-receptor can occur. We found that poliovirus receptor-related protein 2 (Prr2) (Eberlé *et al.,* 1995) (designated here as herpesvirus entry protein B or HveB) was capable of mediating entry into CHO cells of all three mutant HSV-I strains, wild-type HSV-2 strains, and pseudorabies virus (PRV) but had only minimal ability to mediate entry of wild-type HSV-1 strains and bovine herpesvirus 1 (BHV-1). HveB is widely expressed in human tissues and cells, including neuronal cells and keratinocytes. In addition, it may be the only co-receptor expressed in certain human fibroblasts such as HEL299 cells, inasmuch as anti-HveB antibodies could protect these cells from infection.

RESULTS

Failure of KOS-Rid1 to infect CHO cells expressing HveA

To test whether the inability of KOS-Rid1 to infect HveA-expressing CHO cells (Montgomery *et al.,* 1996) was due solely to the known mutation in its gD gene (Dean *et al.,* 1994), we prepared two virus stocks that were genotypically gD-negative but complemented with different forms of gD. KOS-gD6 virus, which has its gD ORF replaced with the *lacZ* gene under control of the CMV promoter, was propagated on complementing Vero cells expressing either wild-type KOS gD or mutant KOS-Rid1 gD to obtain the two virus stocks. The stocks were used to inoculate human HEp-2 cells, HveA-expressing CHO cells (CHO-HVEM-12), or control CHO cells (CHO-C8) and viral entry monitored by quantitating β-galactosidase expression. The virus complemented with wild-type KOS gD was able to infect both HEp-2 and CHO-HVEM-12 cells, but not CHO-C8 cells, whereas the virus complemented with KOS-Rid1 gD could infect only the HEp-2 cells (Fig. 1). Similar results were obtained with strains KOS/tk12 and KOS-Rid1/tk12, which are recombinants of the original strains modified by the insertion of a lacZ gene into the viral thymidine kinase gene (data not shown). The results in Fig. 1 demonstrate that the form of gD expressed is the sole determinant of the difference in ability of KOS and KOS-Rid1 to enter cells via HveA, consistent with the finding that KOS gD binds to HveA, whereas binding of KOS-Rid1 gD to HveA could not be detected (Whitbeck *et al.,* 1997). Because KOS gD differs from KOS-Rid1 and KOS-Rid2 gDs only at amino acid 27, substitutions at this site must account for the mutant phenotypes. Virus expressing the KOS-Rid1 form of gD had reduced ability, compared with virus expressing KOS gD, to infect human T lymphocytes as well as CHO-HVEM-12 cells (Montgomery *et al.,* 1996), whereas viruses expressing either form of gD have a nearly equivalent ability to infect HeLa cells (data not shown) as well as HEp-2 cells (Fig. 1). Thus, the mutant viruses offered the opportunity to screen a HeLa cell cDNA library for plasmids expressing herpesvirus entry proteins other than HveA.

Cloning and identification of a co-receptor for KOS-Rid1 entry

The HeLa cell cDNA expression library used previously to clone HveA (Montgomery *et al.,* 1996) was

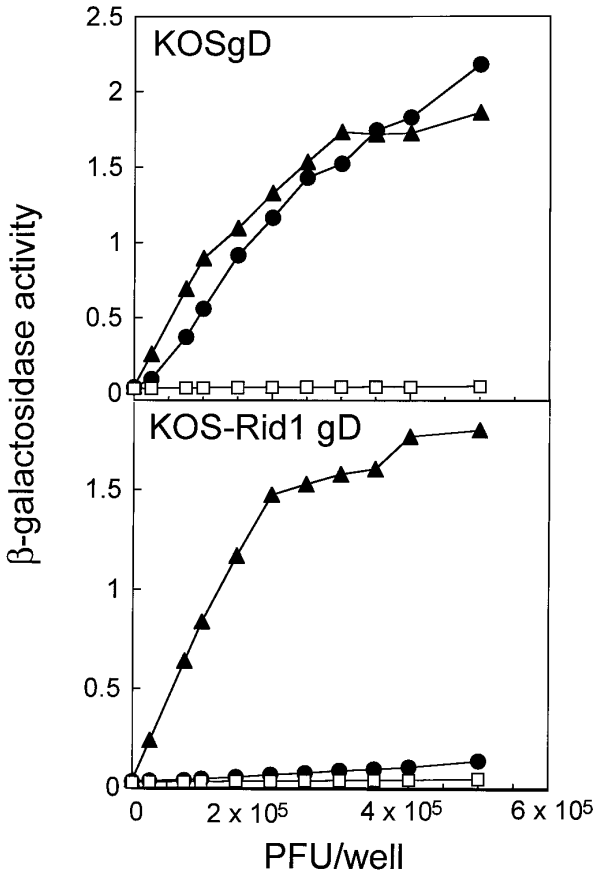

FIG. 1 Rid1 mutation in gD blocks HSV-1 entry into HveA-expressing CHO cells. Hep-2 cells (closed triangles), HveA-expressing CHO-HVEM-12 cells (closed circles), and control CHO-C8 cells (open squares) in 96-well plates were inoculated with KOS-gD6 complemented with KOS gD (top) or with KOS-Rid1 gD (bottom). After 6 h, the cells were permeabilized and substrate (ONPG) was added for quantitation of β-galactosidase activity as a measure of viral entry. The values shown are optical density at 410 nm. Each point is the mean of triplicate determinations for this and the following figures.

screened again by transfecting CHO-K1 cells with plasmid mixtures from the library and then challenging the cells with KOS-Rid1/tk12 to identify susceptible cells by β-galactosidase expression. The transfected/inoculated cells were incubated with X-gal and the monolayers scored for numbers of blue cells. The numbers of cells susceptible to KOS-Rid1/tk12 infection in monolayers transfected with the control plasmid were about 8–13 per 35-mm dish, whereas one of the mixtures of plasmid pools from the cDNA library converted about 95 cells in the monolayer to susceptibility to KOS-Rid1/tk12 infection. The pools in this mixture were individually screened to identify the one with highest entry activity. This pool was further subdivided into 100 pools and the process of screening for entry activity repeated until a single plasmid with activity was isolated. This plasmid, designated pMW347, had a cDNA insert of 1901 base pairs excluding the poly(A) tail.

Sequencing of the plasmid revealed that it encoded a protein nearly identical to poliovirus receptor-related protein 2α (Prr2α), previously cloned on the basis of homology to members of the poliovirus receptor subfamily of the immunoglobulin superfamily. Prr2α and Prr2δ are two membrane glycoproteins expressed from the same locus and transcript by differential splicing after codon 347 (Eberlé *et al.,* 1995). Figure 2 shows the main features of the protein encoded by pMW347, here designated HveB. The sequence determined for the HveB (Prr2α) open reading frame (479 amino acids) is identical to that published for Prr2α (478 amino acids) except for the presence of three additional bases following codon 351 such that codons 352 and 353 of Prr2α (CCT CGC encoding ProArg) become codons 352, 353, and 354 of HveB (GCC TCG CCC encoding AlaSerPro).

Susceptibility of cells expressing HveB to entry of various alphaherpesviruses

CHO-K1 cells either transiently or stably transfected with an HveB-expressing or control plasmid were inoculated with recombinant viruses carrying *lacZ* cassettes so that susceptibility of the cells to infection could be assessed by β-galactosidase expression. Alternatively, CHO-IEβ8 cells were transfected with an HveB-expressing or control plasmid and inoculated with nonrecombinant viruses. CHO-IEβ8 cells carry the *lacZ* gene under control of the HSV-1 ICP4 promoter, so that expression of β-galactosidase is turned on by entry of an HSV strain

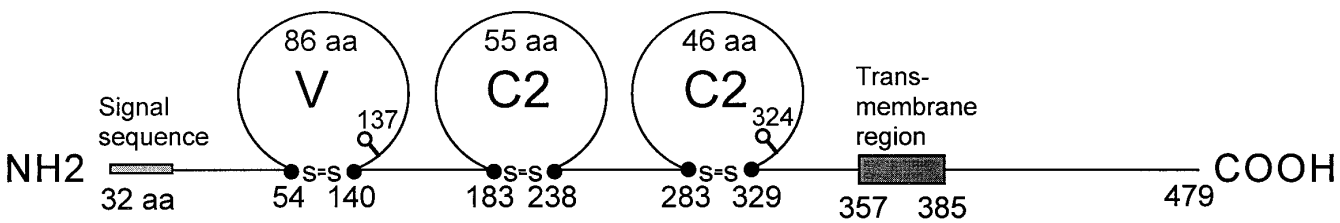

FIG. 2. Features of HveB. The 479-amino-acid translation product has an N-terminal signal sequence, three immunoglobulin-like domains in the extracellular portion (V, C2, C2), two potential sites for the addition of N-linked glycans (lollipops at positions 137 and 324), a hydrophobic domain that presumably spans the membrane, and a cytoplasmic tail of 94 amino acids. The amino acid sequence deduced from nucleotide sequence of the plasmid encoding HveB (GenBank Accession No. AF058448) was nearly identical to that published for Prr2-α (Eberlé *et al.,* 1995) except as noted in the text.

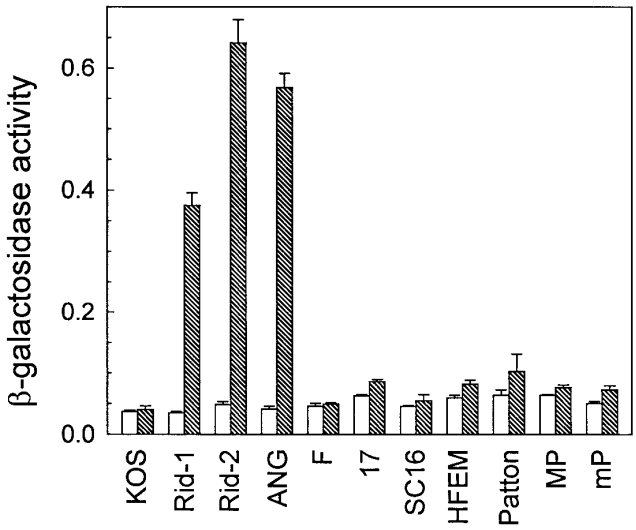

FIG. 3. Susceptibility of HveB-expressing CHO cells to entry of various HSV-1 strains. CHO-IEβ8 cells were transfected with HveB-expressing pMW347 (hatched bars) or empty vector pcDNA3 (open bars), plated in 96-well plates and then inoculated with serial dilutions of the viruses indicated. After 6 h, the cells were permeabilized and incubated with ONPG substrate for quantitation of β-galactosidase activity as described in Fig. 1 and the text. The results depicted show entry of virus at a single input dose of 7.5×10^4 PFU per well in the linear range of the dose–response curve for each virus. The error bars indicate standard deviation of triplicate determinations.

that releases the regulatory protein VP16 from its tegument into the cell. Figure 3 shows results obtained after inoculation of transfected CHO-IEβ8 cells with a variety of HSV-1 strains. The HveB-expressing cells (hatched bars) were susceptible to entry of the mutant strains KOS-Rid1 and KOS-Rid2 and strain ANG but remained almost as resistant as the control-transfected cells (open bars) to entry of the other HSV-1 strains tested. Results similar to those shown in Fig. 3 have also been obtained using transiently or stably transfected HveB-expressing CHO cells and β-galactosidase-expressing recombinants of KOS and KOS-Rid1 (data not shown).

To determine whether HveB could mediate infection by other alphaherpesviruses, HveB-expressing or control CHO-IEβ8 cells were inoculated with HSV-2 strains 333 and WTW1A and HveB-expressing or control CHO-K1 cells were inoculated with β-galactosidase-expressing recombinants of BHV-1 and PRV. The results shown in Fig. 4 demonstrate that HveB expression enhanced the entry of HSV-2 and PRV but had little effect on BHV-1 entry. Similar results were obtained over a range of input doses of virus, one of which within the linear range of the dose response for each virus is shown in Fig. 4. Although control CHO cells are partially susceptible to HSV-2 entry (Shieh *et al.,* 1992), as is evident from Fig. 4, enhancement of viral entry was readily observed in HveB-expressing cells for some, but not all, HSV-2 strains tested. Lack of enhancement correlated with very high levels of infection of the control CHO cells, suggesting that HveB could not contribute appreciably when entry activity was already very high in its absence. Whether HveB can independently mediate HSV-2 entry or facilitates entry via natural co-receptors for HSV-2 expressed by CHO cells remains to be determined. The former alternative seems more likely, given the results obtained with HSV-1 mutants and PRV.

Previous results (Montgomery *et al.,* 1996) and those presented here show that both HveA and HveB enhanced HSV-2 infection of CHO cells. However, HveA mediated entry of wild-type HSV-1 strains but not the mutants with amino acid substitutions at amino acid 27 in gD while the converse is true for HveB. HveA had no demonstrable activity in promoting entry of PRV or BHV-1 (Geraghty *et al.,* 1998), whereas HveB can mediate PRV entry. In a related study we have shown that poliovirus receptor-related protein 1 (Prr1), designated HveC, mediated entry of all the alphaherpesviruses mentioned above and poliovirus receptor (Pvr), also designated Pvr-HveD, mediated entry of PRV and BHV-1 (Geraghty *et al.,* 1998).

Expression of HveB in various cell types

To detect HveB mRNA in total RNA extracted from various cultured cell types, reverse transcription followed by PCR was performed, using primers that

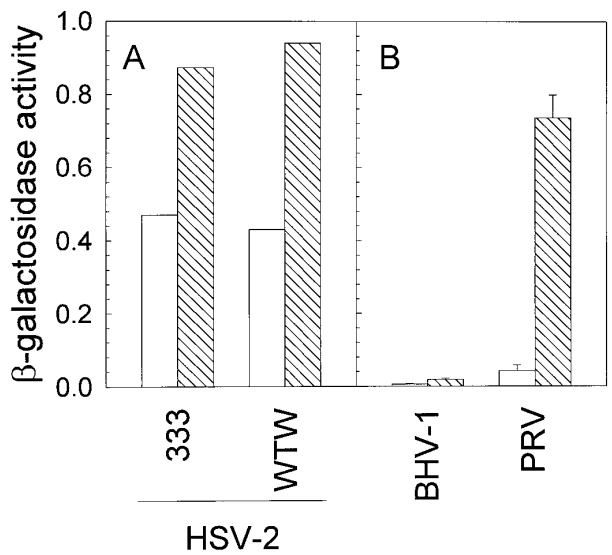

FIG. 4. Susceptibility of HveB-expressing CHO cells to HSV-2, BHV-1, and PRV. Cells in 96-well plates were inoculated with serial dilutions of HSV-2(333), HSV-2(WTW1A), or β-galactosidase-expressing recombinants of BHV-1 or PRV. (A) The cells were CHO-IEβ8/HveB (hatched bar) and CHO-IEβ8 (open bar); (B) the cells were CHO-K1 transiently transfected with HveB-expressing pMW20 (hatched bars) or empty vector pcDNA3 (open bars). After 6 h, the cells were permeabilized and incubated with ONPG substrate for quantitation of β-galactosidase activity as described in Fig. 1 and the text. The results depicted show entry of virus at a single input dose in the linear range of the dose–response curve for each virus (3×10^4 PFU/well for both HSV-2 strains, 10^7 PFU/well for BHV-1 and PRV). Similar results were obtained in at least two additional experiments for each virus.

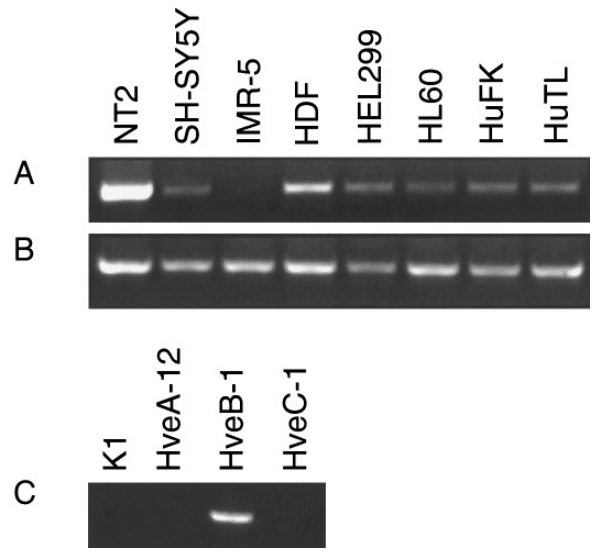

FIG. 5. Expression of HveB mRNA in various cell types. Total RNA was isolated from the cells indicated, cDNAs were produced, and PCR was performed using primers specific for HveB mRNA (A and C) or β-actin mRNA (B). The products were separated by electrophoresis on an agarose gel and then stained with ethidium bromide. Human cells passaged only a limited number of times in culture included diploid fibroblasts (HDF), embryonic lung fibroblasts (HEL299), foreskin keratinocytes (HuFK), and T lymphoblasts (HuTL). Human cell lines included teratocarcinoma (NT2), neuroblastoma (SH-SY5Y and IMR-5), and promyelocytic leukemia (HL60). Also shown are results obtained with RNA extracted from control CHO-K1 cells and stable transfectants expressing HveA, HveB, or HveC (C). The β-actin controls in B were also presented in a figure published elsewhere (Geraghty *et al.,* 1998) that documents expression of HveC mRNA in these cell types.

spanned three exons of the *hveB* gene and would generate a product specific for spliced transcripts from the *hveB* locus. A product of the predicted size for the amplified HveB mRNA sequence was detected in HveB-expressing CHO cells, but not in HveA- or HveC-expressing CHO cells, as expected (Fig. 5C). This product, indicative of HveB expression, was detected in several human cell lines, including NT2 (teratocarcinoma), SH-SY5Y (neuroblastoma), human diploid fibroblasts, HEL299, HL60, human foreskin keratinocytes, and phytohemagglutinin-stimulated human T lymphoblasts (Fig. 5A). HveC mRNA was detected in all of these cells except for the T lymphoblasts and HEL299 cells (Geraghty *et al.*, 1998). It is of interest that one neuroblastoma cell line (SH-SY5Y) expressed both HveB and HveC mRNAs whereas another (IMR-5) expressed only HveC mRNA. Neither HveC nor HveA mRNA (data not shown) could be detected in HEL299 cells, whereas HveB mRNA was detected, suggesting that these cells might be resistant to KOS entry and susceptible to KOS-Rid1 entry via HveB.

Infection of HEL299 cells by KOS-Rid1 and blocking of infection by anti-HveB antibodies

An HveB-expressing CHO cell line (CHO-HveB-1) and HEL299 cells were inoculated with serial dilutions of KOS-gD6 complemented with KOS gD or KOS-Rid1 gD and viral entry assessed by quantitation of β-galactosidase activity. As expected, the HveB-expressing CHO cells were susceptible to KOS-gD6 complemented with KOS-Rid1 gD but not with KOS gD. Similar results were obtained with the HEL299 cells (Fig. 6), whereas other cells including HEp-2 (Fig. 1), HT1080, and HveC-expressing CHO cells (data not shown) were susceptible to KOS-gD6 complemented with either form of gD.

A rabbit antiserum was raised against a truncated form of HveB secreted from baculovirus vector-infected insect cells. Antibodies from the immune serum, but not pre-immune serum, specifically bound to HveB-expressing CHO cells and not to control CHO cells or HveC-expressing CHO cells (Fig. 7). Because HveB and HveC are related in structure, it was important to determine whether the anti-HveB antibodies cross-reacted with HveC. No evidence for cross-reaction was observed. Serial dilutions of the antiserum and control serum were

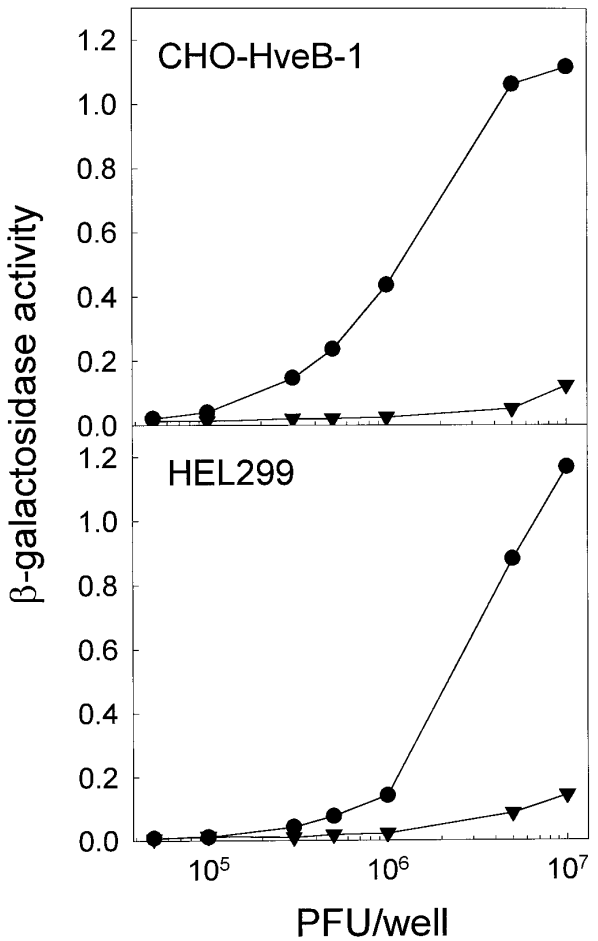

FIG. 6. Susceptibility of HveB-expressing CHO cells and HEL299 cells to KOS-gD6 complemented with KOS-Rid1 gD (circles) but not with KOS gD (triangles). The cells in 96-well plates were inoculated with virus at the input doses indicated and, 6 h later, the cells were permeabilized and ONPG substrate added for quantitation of β-galactosidase activity.

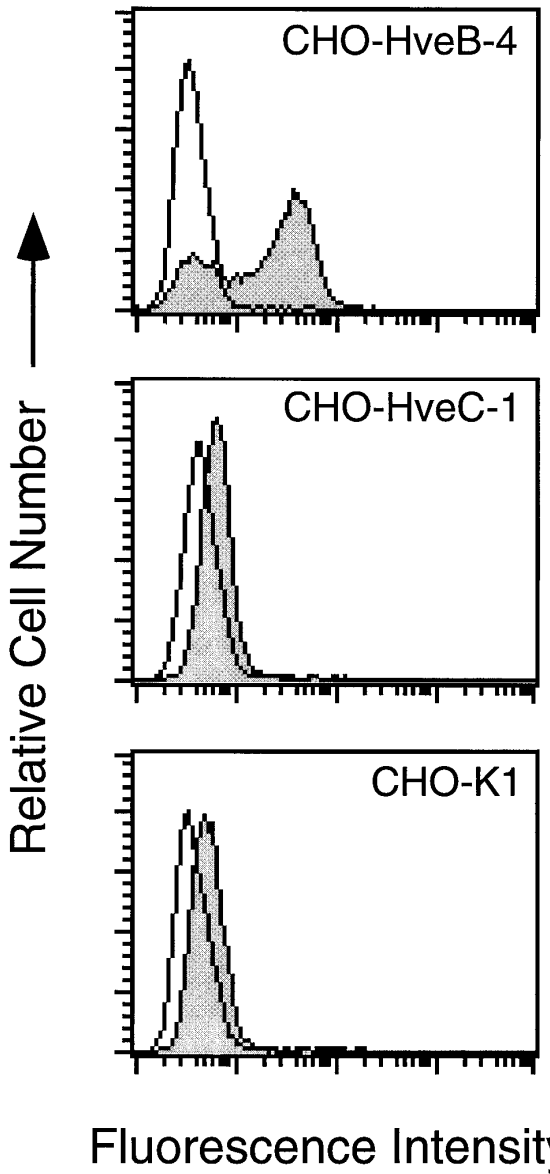

FIG. 7. Anti-HveB antibodies recognize cell surface HveB but not HveC. CHO-HveB-4, CHO-HveC-1, and control CHO-K1 cells were detached and incubated with anti-HveB serum R146 (shaded curves) or with control preimmune serum (open curves), followed by fluorescein-ated anti-rabbit antibodies. Fluorescence intensity as determined by flow cytometry is shown. Similar results were obtained with four other stably transfected CHO-HveB cell lines including CHO-HveB-1 (data not shown).

incubated with CHO-HveB-1 cells and HEL299 cells followed by the addition of KOS-Rid1/tk12 virus. Viral entry into both cell types was almost completely inhibited by the antiserum in dose-dependent fashion, whereas the preimmune serum had no effect or, in the case of HEL299 cells, actually enhanced entry or events leading to β-galactosidase expression (Fig. 8). Enhancement of HSV-1 infection by unknown factors in normal rabbit serum was also noted for human T lymphoblasts (Montgomery *et al.,* 1996) and is currently unexplained. In the face of this enhancing activity in rabbit serum, the inhibition of infection of HEL299 cells by the antiserum is probably even more pronounced than is evident from the simple dose–response curve (Fig. 8).

DISCUSSION

Use of KOS-Rid1 to screen for a mediator of viral entry led to identification of Prr2 (Eberlé *et al.,* 1995) or HveB, which has entry activity for mutants of HSV-1 altered in gD, and for HSV-2 and PRV. Because HveB is closely related to Pvr (Mendelsohn *et al.,* 1989) and Prr1 (Lopez *et al.,* 1995), we also assessed herpesvirus entry activity of the latter two proteins in another study (Geraghty *et al.,* 1998). We found that Prr1 (HveC) could mediate entry of HSV-1 strains (wild-type and mutants altered in gD),

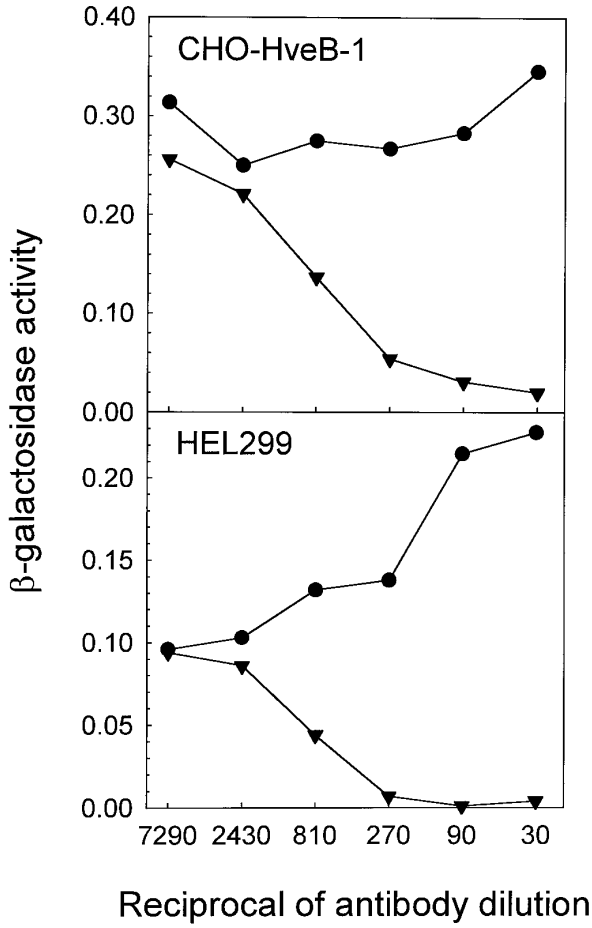

FIG. 8. Anti-HveB antibodies blocked entry of virus into CHO-HveB-1 cells and HEL299 cells. The cells in 96-well plates were incubated for 30 min with serial dilutions of antiserum R146 (triangles) or preimmune serum (circles) and then a constant amount of KOS-Rid1/tk12 virus was added (5×10^5 PFU/well for CHO-HveB-1 cells and 5×10^6 PFU/well for HEL299 cells). After 2 h of incubation, the virus–serum mixtures were removed, unpenetrated virus was inactivated by brief treatment with low pH buffer, and incubation continued for another 4 h. The cells were permeabilized and ONPG substrate added for quantitation of β-galactosidase activity.

TABLE 1

Alphaherpesvirus Entry Proteins and Activity for Mediating Entry of Animal and Human Viruses

Virus	Alphaherpesvirus entry protein (protein family)			
	HveA (TNF-R)[a]	HveB (Ig)	HveC (Ig)[b]	Pvr-HveD (Ig)[b]
HSV-1	+ +	− −	+ + +	− −
HSV-1-Rid[c]	− −	+ +	+ + +	− −
HSV-2[d]	+ +	+ +	+ +	??[d]
PRV	− −	+ +	+ +	+ +
BHV-1	− −	− −	+ +	+ +

[a] From results published by Montgomery *et al.* (1996) and Geraghty *et al.* (1998).

[b] From results published by Geraghty *et al.* (1998).

[c] Mutants of HSV-1 with amino acid substitutions at position 27 in gD (Q27P or Q27R).

[d] Comparisons of HSV-2 entry activity with activity for entry of the other viruses is complicated by the partial or full susceptibility of control CHO cells to HSV-2 entry. Rigorous assessments of comparative activity will have to await use of other cell lines that are as resistant to HSV-2 as to the other viruses.

HSV-2 strains, PRV, and BHV-1 and that Pvr (Pvr-HveD) could mediate entry of PRV and BHV-1. Thus, the three known human members of the immunoglobulin superfamily that include or are closely related to the Pvr are able to mediate entry of one or more of the human and animal alphaherpesviruses used in these studies (Table 1). Genes for two of these proteins (Pvr-HveD and HveB) are located on chromosome 19 (Eberlé *et al.,* 1995; Koike *et al.,* 1990), whereas HveC is located on chromosome 11 (Lopez *et al.,* 1995). The gene for HveC may have been responsible for the observed ability of chromosome 11 to confer susceptibility to HSV infection on Chinese hamster lung–human hybrid cells (Francke and Francke, 1981).

It is somewhat surprising that a member of the TNF receptor family (HveA) as well as members of the immunoglobulin superfamily (HveB and HveC) can mediate entry of HSV strains, especially because it appears that gD may be the viral ligand for all these receptors. Evidence has been reported of direct physical interaction between the ectodomains of HveA and either HSV-1 or HSV-2 gD (Whitbeck *et al.,* 1997) and of binding of soluble HveA to virions via interaction with gD (Nicola *et al.,* 1998). Similar evidence for interaction between the ectodomains of HveC and gD has also been obtained (C. Krummenacher, A. V. Nicola, J. C. Whitbeck, H. Lou, W. Hou, J. D. Lambris, R. J. Geraghty, P. G. Spear, G. H. Cohen, and R. J. Eisenerg, 1998, Submitted for publication). Moreover, the allele of gD expressed determines whether HSV-1 can enter cells via HveA or HveB. Viruses carrying the wild-type form of gD can infect HveA-expressing cells, but not HveB-expressing cells, whereas the converse is true for viruses carrying the Rid mutant forms (Table 1). In the case of HveA, the fact that HveA can bind to wild-type gD but not to Rid forms of gD provides the basis for the observed specificity (Whitbeck *et al.,* 1997). It remains to be determined whether the specificity exhibited by HveB is also determined by direct interactions with HSV-2 gD and the mutant forms of HSV-1 gD, but not wild-type HSV-1 gD.

Although the PRV and BHV-1 ligands for HveB and HveC have not been identified, it seems likely that they will be members of the gD family expressed by these viruses. The forms of gD expressed by PRV and BHV-1 exhibit only 10 to 15% sequence identity with HSV-1 or HSV-2 gD. Nevertheless, a gD domain capable of interacting with HveB may be conserved among the HSV-1 Rid mutants, HSV-2 strains, and PRV and another distinct gD domain capable of interacting with HveC may be conserved among HSV-1, HSV-2, PRV, and BHV-1. Our results identify human cell surface proteins by which these human and animal viruses could penetrate specific human cells but do not speak to other postentry requirements for productive or latent infection.

The fact that PRV can use HveB, HveC, and Pvr-HveD, and BHV-1 can use HveC and Pvr-HveD, for entry into CHO cells strongly suggests that porcine and bovine homologs of these human cell surface proteins may be functional entry proteins for these animal viruses in cells of the natural host. Homologs of this human subfamily of the immunoglobulin superfamily have been identified in monkeys, mice, and rats (Chadeneau *et al.,* 1994; Koike *et al.,* 1992; Morrison and Racaniello, 1992). Work in progress (D. Shukla and P. G. Spear, manuscript in preparation) demonstrates that the mouse homolog of HveB, also known as the murine poliovirus receptor homolog (Mph) (Morrison and Racaniello, 1992), can mediate the entry of PRV, suggesting that alphaherpesviruses have evolved to interact with highly conserved domains and/or multiple domains of proteins in this receptor subfamily.

Our proposal for the nomenclature of alphaherpesvirus entry proteins is based on several considerations. First, the names Prr1 and Prr2 are misleading because these proteins are related to Pvr by sequence but not by functional activity in poliovirus entry. All three proteins can serve as alphaherpesvirus entry proteins. Second, research on the human and animal forms of the multiple alphaherpesvirus entry proteins will be facilitated by the assignment of a unique name to each gene encoding one of these entry proteins (*hveA, hveB, hveC, Pvr-HveD*), with use of protein names (HveA, HveB, HveC, Pvr-HveD) that can be modified as needed to identify the species of origin (human HveB and mouse HveB, for example). Clearly, the cross interactions of alphaherpesviruses with entry proteins of the natural and other hosts will be easier to track under this system of nomenclature. Finally, this nomenclature follows the conventions for naming genes and proteins of multiple loci that encode proteins with apparently equivalent functions (for alphaher-

pesvirus entry, at least). Obviously, other functions for these entry proteins will be defined in the future, at which time changes in, or additions to, the names may be appropriate.

Human HveB, HveC, and Pvr-HveD and the animal homologs studied to date are expressed in many cells and organs of the intact organism and in a variety of cultured cell types. Two membrane-bound forms of HveB, differing in their membrane-spanning regions and cytoplasmic tails, have been identified (Eberlé *et al.,* 1995). Also, multiple membrane-bound and secreted forms of Pvr-HveD have been identified (Koike *et al.,* 1990; Mendelsohn *et al.,* 1989). Little is known about the normal physiological roles of any of these proteins. The mouse homolog of HveB has been reported to bind to itself and to mediate homophilic cell aggregation, suggesting that it is a homophilic intercellular adhesion molecule (Aoki *et al.,* 1997). Interestingly, five independently derived stable CHO transfectants cloned for expression of HveB evolved into populations expressing both low and high levels of cell surface HveB (see Fig. 7). If human HveB has the same activity as mouse HveB, perhaps high-level expression of HveB by all cells in the population cannot be maintained because of deleterious effects of intercellular adhesion.

Results presented here and in a related study (Geraghty *et al.,* 1998) indicate that HveB and HveC can be expressed individually or together in various human cell types of neuronal, fibroblastic, and epithelial origin. In these cell types, either or both of these proteins could be important entry mediators for HSV-2 strains and rare variant HSV-1 strains, whereas wild-type HSV-1 strains would be able to use HveC but not HveB. In fact, KOS-Rid1 but not KOS could infect a human fibroblastic cell strain, HEL299, via HveB in that antibodies specific for HveB protected these cells from infection. It seems likely that the variable ability of different HSV-1 and HSV-2 strains to utilize HveA, HveB, and HveC (and other unidentified proteins) for entry and variable expression of these entry proteins in different cell types will govern in large part any strain-specific and serotype-specific patterns of viral spread in infected persons. Any polymorphisms in the genes encoding these proteins might also contribute to human differences in susceptibility to infection and disease.

MATERIALS AND METHODS

Cells and viruses

Cell lines used included CHO-K1, HEp-2, Vero, HT1080, NT2 (Andrews *et al.,* 1984), SH-SY5Y (Ross *et al.,* 1983), IMR-5 (Gilbert and Malenbaum, 1980), and HL60. Human cells passaged only a limited number of times from primary isolation included diploid lung fibroblasts HEL299 obtained from the American Type Culture Collection (Peterson *et al.,* 1968), diploid fibroblasts obtained from M. K. Rundell, foreskin keratinocytes obtained from L. Laimins (Thomas and Laimins, 1998), and T lymphoblasts (Montgomery *et al.,* 1996). Stable transfectants of CHO-K1 cells expressed HveA (CHO-HVEM-12) (Montgomery *et al.,* 1996), HveB (CHO-HveB-1 and CHO-HveB-4), or HveC (CHO-HveC-1) (Geraghty *et al.,* 1998) or maintained the empty vector pcDNA3 (CHO-C8) (Montgomery *et al.,* 1996). CHO-HveB-1 and -4 cell clones were transfected with pMW20 as described below. Another stable CHO-K1 transfectant (CHO-IEβ8) maintained a plasmid with the *lacZ* gene under control of the HSV-1 ICP4 promoter; expression of β-galactosidase was not constitutive but was induced by entry of the tegument transactivator VP16 upon infection by HSV (Montgomery *et al.,* 1996). Using methods described previously (Terry-Allison *et al.,* 1998), CHO-IEβ8 cells were stably transfected with HveB-expressing pMW20 to yield CHO-IEβ8/HveB cells, which were susceptible to infection by some HSV strains through constitutive expression of HveB and remained inducible for expression of β-galactosidase upon HSV infection. Stable transfectants of Vero cells, designated Vero15-D1 and Vero14-Rid1, were inducible for KOS gD or KOS-Rid1 gD expression, respectively, by infection with HSV. The plasmids used to produce these complementing cell lines carried the HSV-1 genes for gJ, gD (wild-type or mutant versions), and gI and the puromycin-resistance marker. For wild-type gD, the insert of pHD15 (Dean *et al.,* 1994) was excised with *Sap*I and *Nde*I and cloned between the same sites in pPUR (Invitrogen). For Rid1 gD, the construction was similar except the insert used was from pHD31 (Dean *et al.,* 1994).

Wild-type HSV-1 strains used included KOS, F, 17, SC16, HFEM and Patton. Wild-type HSV-2 strains were 333 and WTW1A. HSV-1(F) (Ejercito *et al.,* 1968) and HSV-2(WTW1A) (Terhune *et al.,* 1998) have been passaged only a limited number of times in HEp-2 cells since isolation. HSV-1 strains MP and mP are syncytial and nonsyncytial strains, respectively, selected as plaque variants under an antibody overlay (Hoggan and Roizman, 1959). HSV-1 mutant strains included KOS-Rid1 and KOS-Rid2, both selected for ability to infect gD-expressing HEp-2 cells and thus for resistance to gD-mediated interference (Dean *et al.,* 1994). Recombinants of KOS, KOS-Rid1, and KOS-Rid2 were engineered to express β-galactosidase under control of the HSV-1 ICP4 promoter; they had the appropriate *Sal*I to *Bam*HI fragment of pON105 (Ho and Mocarski, 1988) inserted between the *Sac*I and *Sph*I sites (changed to *Bam*HI and *Sal*I sites) of the viral thymidine kinase gene, replacing base pairs 47,359 to 47,412 of the viral genome, and designated KOS/tk12, KOS-Rid1/tk12, and KOS-Rid2/tk12, respectively. A gD-negative KOS recombinant, designated KOS-gD6, was engineered to contain the *lacZ* gene under control of the CMV promoter inserted between the *Hin*dIII site just upstream of the gD ORF and the gD stop codon, replacing base pairs 138,349 to

139,600 of the viral genome. KOS-gD6 was propagated on Vero15-D1 cells for complementation with KOS gD and on Vero14-Rid1 cells for complementation with KOS-Rid1 gD. HSV-1(ANG) was isolated from a lesion on the thigh by plating under an agar overlay (Munk and Donner, 1963). Animal herpesviruses used were gH-negative PRV(Kaplan), provided by T. Mettenleiter, and BHV-1(Cooper)v4a, provided by L. Bello. The PRV recombinant expressed β-galactosidase under control of the PRV gG promoter from an insert within the gH gene and was propagated and titered on complementing gH-expressing VeroSW78 cells (Babic *et al.,* 1996). BHV-1(Cooper)v4a, which expressed β-galactosidase under control of the BHV-1 gB promoter from an insert within the viral thymidine kinase gene, was propagated and titered on MDBK cells (Miller *et al.,* 1995). Except as noted otherwise, virus strains were propagated on HEp-2 cells and titered on Vero cells.

Screening assay for plasmids encoding HSV entry proteins

Bacteria containing a HeLa cell cDNA expression library (Invitrogen Corp., Catalog No. A950-10) had previously been divided into 100 pools and frozen as glycerol stocks as described (Montgomery *et al.,* 1996). Samples of each pool were combined into groups of 10 and grown in LB medium with ampicillin and tetracycline and plasmids were prepared by standard methods. The plasmid preparations and pcDNA3 as a control empty vector were transfected into CHO-K1 cells using LipofectAMINE reagent (Gibco-BRL) according to the manufacturer's instructions. At 24–36 h after transfection, the cells were inoculated with KOS-Rid1/tk12 virus at 5 PFU per cell and, 6 h later, were fixed and stained with X-gal as described (Montgomery *et al.,* 1996). The number of blue cells per 35-mm well was counted for each transfection and averaged for duplicates. Individual stocks in the plasmid group with greatest activity were tested to identify the stock with highest activity. This stock was subdivided by plating samples on 100 100-mm plates at about 1000 colonies per plate. The colonies on each plate were pooled and the process described above repeated until a single plasmid, designated pMW347, was obtained. The insert of pMW347 was excised with *Hin*dIII and *Xba*I and transferred to pcDNA3 to obtain pMW20, which carried both the insert and a Neo selectable marker. Nucleotide sequence of the insert was determined by the University of Chicago Cancer Research Center Sequencing Facility, using a UBI Prism 377 DNA sequencer. The GenBank accession number is AF058448.

Entry assays

Cells were plated in 96-well plates and, after overnight incubation, were inoculated with virus serially diluted in phosphate-buffered saline containing 0.1% glucose and 1% calf serum (PBS-G-CS) and incubated at 37°C. When antibodies were tested for the ability to protect cells from infection, serial dilutions of antiserum prepared in PBS-G-CS were incubated with the cells for 30 min at 37°C followed by the addition of virus. After 2 h of incubation with the virus–antiserum mixtures, the mixtures were removed and the cells treated with 0.1 M citrate buffer (pH 3) to inactivate unpenetrated virus and then washed. PBS-G-CS was added for continued incubation. Recombinant viruses expressing β-galactosidase were used for all cell types except CHO-IEβ8. Because CHO-IEβ8 cells express β-galactosidase from a cell-associated reporter gene upon entry of HSV, viruses without reporter genes could be used on these cells. Six hours after the addition of virus, the cells were washed and incubated with the β-galactosidase substrate *O*-nitrophenyl β-d-galactopyranoside (ONPG), dissolved at 3 mg/ml in phosphate-buffered saline containing 0.5% NP-40. At various times after adding substrate, the plates were read at 410 nm in a Spectra Max 250 ELISA reader.

Detection of mRNAs by RT-PCR

Total RNA was isolated from 1×10^6 to 5×10^6 cells using the RNeasy kit (Qiagen). The 3′ RACE kit (Gibco-BRL) was used for reverse transcription. PCR amplification of cDNAs was done with primers PRR2A8 (5′AGAAGCAGCAGCACCAGCAG) and PRR2A9 (5′AAGGTCACGTTCAGCCAGGA) for HveB and HVEM228 (5′ATCATATGTGTGAAAAGAAGA) and HHVENT03 (5′CAGGTTATCGTGTGAAGGAG) for HveA. The β-actin control primers and thermocycling conditions have been described (Willey *et al.,* 1996). The PCR products were analyzed by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

Construction of the baculovirus recombinant expressing HveB(361t)

The general strategy was as described for construction of a baculovirus recombinant expressing a secreted form of HSV-1 gD (Sisk *et al.,* 1994). The ectodomain of HveB minus its signal peptide was obtained by PCR amplification using primers 5′-GCGAGATCTGCGAGTTCAAGTGCTA and 5′-GCGTGATCAGTGGTGATGATGGTGATGCACCAGCGGACCCACATCTC. The PCR product was digested with *Bgl*II and *Bcl*I (sites indicated by boldface letters) and inserted into the *Bam*HI site of pVT-Bac to generate pCW284. pVT-Bac fuses a mellitin signal sequence to the N-terminus of an inserted coding region and a histidine hexamer to the C-terminus. The plasmid pCW284 was cotransfected with baculovirus DNA (Baculogold, Pharmingen) into Sf9 cells growing in monolayer culture. Viral progeny were screened for expression of the secreted recombinant protein HveB(361t) by Western blot with R143 antiserum and positive clones were plaque-purified. Bac-HveB(361t) was the viral re-

combinant used routinely for preparation of purified HveB(361t). This recombinant protein comprising amino acids 33 to 361 of the HveB ectodomain was purified from the medium of infected Sf9 cells by chromatography on nickel-NTA resin (Qiagen), dialyzed against PBS, and concentrated as described previously (Sisk *et al.,* 1994).

Antibodies and flow cytometry

Antiserum R143 was generated by immunizing a rabbit with KLH coupled to a 22-amino-acid peptide (DVRVQV-LPEVRGQLGGTVELPC) representing a hydrophilic portion of the HveB ectodomain. This antiserum recognized the peptide used for immunization by dot blot assay and HveB(361t) by Western blot. Antiserum R146 was generated by immunization of a rabbit with purified HveB(361t) as previously described for HVEM(200t) (Terry-Allison *et al.,* 1998). Specific binding of antibodies (antiserum R146) to HveB on cell surfaces was assessed by flow cytometry. The antiserum and preimmune serum were diluted 1:100 and incubated with the cells. After washing the cells were incubated with a 1:200 dilution of fluorescein-ated goat anti-rabbit antibodies and analyzed on a Becton–Dickinson FACScan.

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