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Herpes Simplex Virus-1 Entry into Cells Mediated by a Novel Member of the TNF/NGF Receptor Family

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Summary

We identified and cloned a cellular mediator of herpes simplex virus (HSV) entry. Hamster and swine cells resistant to viral entry became susceptible upon expression of a human cDNA encoding this protein, designated HVEM (for herpesvirus entry mediator). HVEM was shown to mediate the entry of several wild-type HSV strains of both serotypes. Anti–HVEM antibodies and a soluble hybrid protein containing the HVEM ectodomain inhibited HVEM–dependent infection but not virus binding to cells. Mutations in the HSV envelope glycoprotein gD significantly reduced HVEM–mediated entry. The contribution of HVEM to HSV entry into human cells was demonstrable in activated T cells. HVEM, the first identified mediator of HSV entry, is a new member of the TNF/NGF receptor family.

Introduction

Herpes simplex viruses (HSV) are human members of the neurotropic subgroup (alphaherpesviruses) of the herpesvirus family. Infections with HSV type 1 (HSV-1) and HSV type 2 (HSV-2) are highly prevalent. The usual manifestations of disease (reviewed by Corey and Spear, 1988) are mucocutaneous lesions of the mouth, face, eyes, or genitalia. Both HSV-1 and HSV-2 establish latent infections in neurons of peripheral ganglia and may reactivate to cause recurrent lesions. Rarely, the virus spreads to the central nervous system to cause meningitis or encephalitis.

Viral infection in the natural host usually remains localized to cells of the epidermis and peripheral nervous system, except in newborn infants, who are more prone to disseminated infection. Differentiated cell types probably vary in their susceptibility to HSV entry. However, cultured cells of various types from many animal species are susceptible to HSV infection, indicating that cell receptors for viral entry may be highly conserved or are multiple in number and are usually expressed on dividing cells.

The binding and penetration phases of HSV entry into cells can be experimentally dissociated. Binding of HSV-1 or HSV-2 to cells is mediated by interaction of viral envelope glycoproteins (gB or gC or both) with glycosaminoglycan chains (GAGs) of cell surface proteoglycans (reviewed by Spear, 1993). Penetration is by pH-independent fusion of the virion envelope with the cell plasma membrane or an early endosome (Wittels and Spear, 1991). Viral glycoproteins required for penetration include gB, gD, and gH–gL hetero-oligomers (Sarmiento et al., 1979; Cai et al., 1988; Ligas and Johnson, 1988; Forrester et al., 1992; Roop et al., 1993). Binding of HSV to cells is not sufficient to mediate penetration. Certain cell types, such as swine testis (ST) or Chinese hamster ovary (CHO) cells, can bind virus efficiently but restrict viral entry (Shieh et al., 1992; Subramanian et al., 1994). Susceptible cells can be made resistant to HSV entry while retaining ability to bind virus by transfection with plasmids expressing HSV gD (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989). These observations suggest that cell-associated gD may sequester or down-regulate a cellular protein required for HSV entry and that ST and CHO-K1 cells fail to express such a mediator of HSV entry.

By screening a human cDNA expression library for genes that could mediate HSV-1 entry into CHO-K1 cells, we identified a previously undescribed member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor family, designated HVEM. This receptor mediated efficient entry of HSV-1 strains into CHO-K1 cells and ST cells and also enhanced the entry of an HSV-2 strain. HVEM was active in mediating HSV-1 entry into human T lymphoblasts.

Results

Cloning a Mediator of HSV-1 Entry

The CHO-K1 cell line is particularly resistant to entry of HSV-1 strain KOS (Shieh et al., 1992), abbreviated here KOS. A β-galactosidase–expressing version of this strain, HSV-1(KOS)gL86 abbreviated here KOS-gL86, was used to monitor viral entry. Production of β-galactosidase from the input viral genome signals that virus has entered the cell, released its genome to the nucleus, and activated the consititutive promoter driving β-galactosidase expression. Figure 1A shows that HeLa (human) cells were susceptible to infection by this virus, whereas CHO-K1 cells were highly resistant. Concentrations of input virus sufficient to infect 100% of HeLa cells left the CHO-K1 cells totally uninfected (Figure 1B).

The strategy to isolate a human cDNA that enhanced HSV-1 entry into CHO-K1 cells combined transient expression of cDNAs with KOS-gL86 challenge. Plasmid DNAs prepared from pools of a HeLa cell cDNA library were transfected into CHO-K1 cells. The cells were exposed to KOS-gL86 and then X-gal to identify infected cells. A pool containing a positive cDNA was subdivided by an iterative process to identify individual plasmid clones that had the desired phenotype.

A set of two plasmid clones (pBEC580 and pBEC748) that rendered transfected CHO-K1 cells susceptible to KOS-gL86 entry (Figures 1B and 1C) were isolated. Nucleotide sequencing of the cDNA insert (the same for both plasmids) revealed a 1698 bp cDNA encoding an open reading frame of 283 amino acids (Figure 2). The protein product, designated HVEM, has characteristics of a type I membrane glycoprotein with an N-terminal signal peptide, two potential sites for addition of N-linked glycans, and a probable membrane-spanning domain.

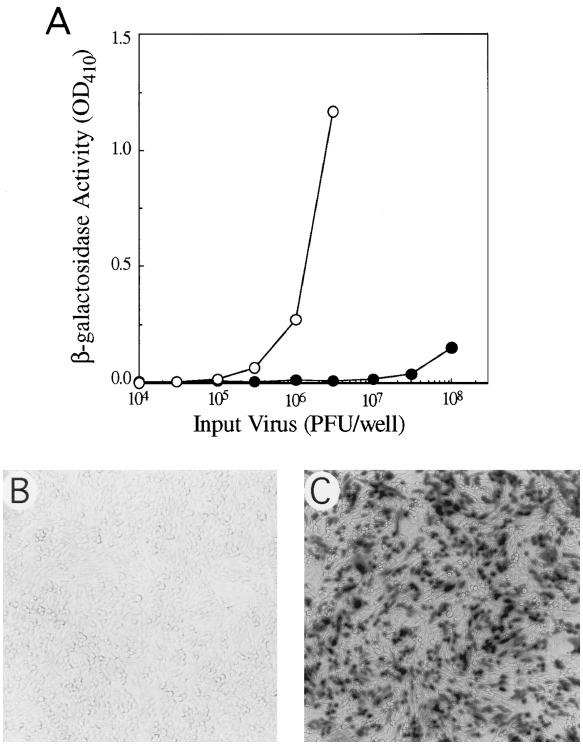

Figure 1. Entry of HSV-1 into HeLa Cells, CHO-K1 Cells, and Transfected CHO-K1 Cells

(A) CHO-K1 cells or HeLa cells were exposed to KOS-gL86 at the doses indicated. After the addition of virus (6 hr), infection was quantitated by monitoring the activity of β-galactosidase expressed from the input viral genome. In this and other figures, each point represents the mean of triplicate determinations. The individual values were within 10% of the mean. HeLa cells (open circles) and CHO-K1 cells (closed circles), values obtained 4 hr after ONPG addition.

(B and C) CHO-K1 cells were transfected with control plasmid pcDNA3 (B) or with pBEC580, isolated from the human cDNA library for its ability to confer susceptibility to HSV-1 infection (C). At 30 hr after transfection, the cells were exposed to KOS-gL86 at 100 pfu/cell. Later (6 hr) the cells were washed, fixed, and incubated with X-gal to identify infected cells.

Searches of protein databases with the predicted HVEM amino acid sequence failed to identify any proteins closely related to HVEM but did identify cysteine-rich repeats in HVEM that are characteristic of the TNF/NGF receptor family (Armitage, 1994). Pair-wise alignments of HVEM sequence with those of other human members of the receptor family revealed sequence identities ranging from 16.9% to 25.4% and similarities ranging from 26.9% to 37.1%. However, cysteines and other residues conserved within the cysteine-rich repeat regions of family members were also conserved in HVEM (residues marked with asterisks in Figure 2). Clearly, HVEM is a new member of the TNF/NGF receptor family.

Southern analyses were performed with HVEM probes and genomic DNAs from various cell lines. Probes from the purified cDNA hybridized to genomic DNA fragments from HeLa and HEp-2 (human) cells, Vero (monkey) cells, and CHO-HVEM12 cells (stably transfected with an HVEM cDNA clone), but not from control CHO-K1 cells (Figure 3). The smaller PvuII probe hybridized to a single DNA fragment from three different human cell lines (HT1080 not shown) and to a single DNA fragment of different size from monkey cells (Figure 3B). The full-length EcoRI probe lacked sites for the restriction endonuclease used to digest the cell DNAs (BamHI) yet hybridized to additional bands in human and monkey DNA samples (Figure 3C). Thus, it is likely that HVEM is encoded by a single-copy human gene with multiple exons and that nonhuman primates have a related gene.

Expression of HVEM

Labeled probes prepared from HVEM cDNA were hybridized to a Northern blot of polyadenylated RNAs extracted from several human tissues. HVEM–related RNAs were detected in most samples tested, with highest levels in lung, liver, and kidney and least in brain (Figure 3E). The RNAs were heterogeneous in size. One species of about 2 kb was similar in size to the HVEM cDNA. The presence of larger species (4–5 kb) suggests that transcription of the gene for HVEM may result in multiple mRNAs.

We produced hybrid and epitope-tagged versions of HVEM to verify expression of the protein and to isolate material for immunization and detection of antibodies. HVEM:Fc, comprising the three-and-a-half cysteine-rich repeats of the ectodomain (see Figure 2) fused in-frame to the Fc region of rabbit IgG heavy chain, was secreted from transfected cells as a glycoprotein (about 50–65 kDa). Changes in size after treatment of HVEM:Fc with glycosidases were consistent with presence of O-linked and complex N-linked carbohydrate chains (Figure 4A). The glycosylation and secretion of HVEM:Fc suggests that the hydrophobic region between amino acids 23 and 38 serves as a signal sequence even though it does not strictly adhere to rules for cleavable signal sequences.

An epitope-tagged version of HVEM was also engineered by fusing HVEM cDNA encoding the first 257 amino acids (see Figure 2) to an oligonucleotide encoding an influenza virus hemagglutinin epitope (Flu epitope). CHO-K1 cells transfected with a plasmid expressing this protein (HVEM-257Flu) produced several Flu-tagged HVEM species ranging in size from about 30 to 90 kDa (Figure 4B). The higher molecular mass species may be multimers of HVEM-257Flu or have bulky posttranslational modifications.

Rabbits were immunized with purified HVEM:Fc and antiserum tested for antibodies specific for HVEM. Cell extracts from CHO-K1 cells transfected with control or HVEM-257Flu-expressing plasmids were immunoprecipitated with rabbit antiserum or control preimmune serum, followed by Western analyses for detection of the Flu epitope. The rabbit antiserum precipitated HVEM-257Flu (lanes 1 and 2) but not a Flu-tagged HSV-1 glycoprotein (lane 3), whereas preimmune serum lacked specific precipitating activity (Figure 4C). Anti–HVEM antibodies preferentially precipitated the higher molecular mass forms of HVEM-257Flu.

HVEM–Mediated Entry of HSV-1 and HSV-2 Strains into Transfected Cells

CHO-K1 and ST cell lines, both resistant to HSV-1 entry, were transfected with control or HVEM–expressing plasmids to obtain stable clones. The HVEM–expressing

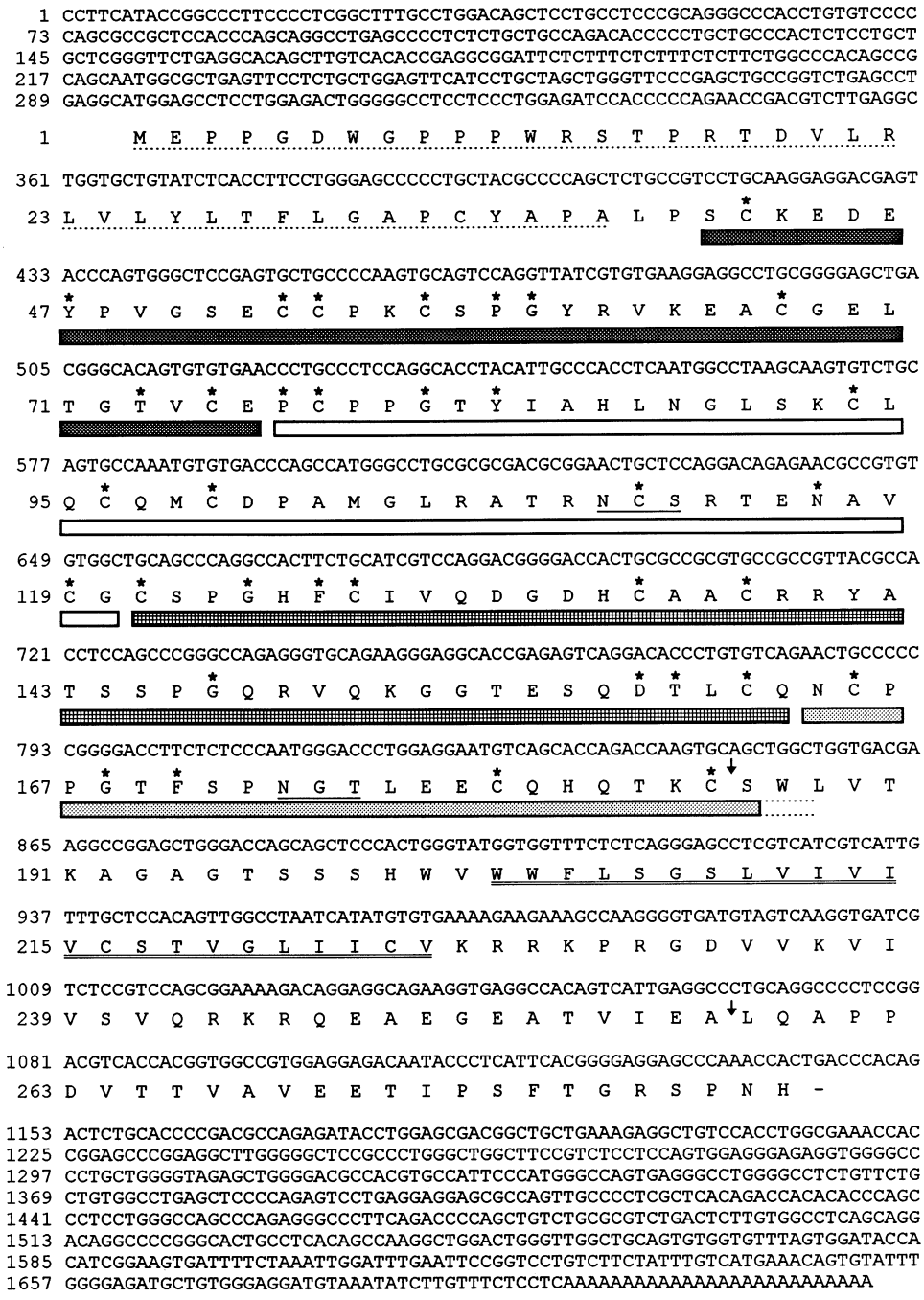

Figure 2. Nucleotide Sequence of the HVEM cDNA and Amino Acid Sequence of the Open Reading Frame

Features of the 283 amino acid open reading frame include a signal peptide (dotted underline), two potential sites for the addition of N-linked carbohydrate (single underline), and the probable membrane-spanning domain (double underline). Also indicated by patterned bars under the amino acid sequence are the three complete and one partial cysteine-rich repeats characteristic of members of the TNF/NGF receptor family. Asterisks show the positions of amino acids that are highly conserved in the family. The arrow after Cys-185 indicates the last amino acid of HVEM present in the fusion protein, HVEM:Fc. The arrow after Ala-257 indicates the last amino acid of HVEM present in the epitope-tagged truncated protein, HVEM257-Flu. The sequence deposited in GenBank was assigned accession number U70321.

clones were 100–1000 times more susceptible to KOS-gL86 infection than control clones (Figure 5A), which were as resistant as the parental cell lines. CHO-K1 cells, but not ST cells, are also resistant to entry of a porcine alphaherpesvirus, pseudorabies virus (PRV). HVEM–expressing clones did not differ from control clones or parental cell lines in susceptibility to infection by a β-galactosidase–expressing mutant of PRV (data not shown). Thus, HVEM is not a general mediator of alphaherpesvirus entry.

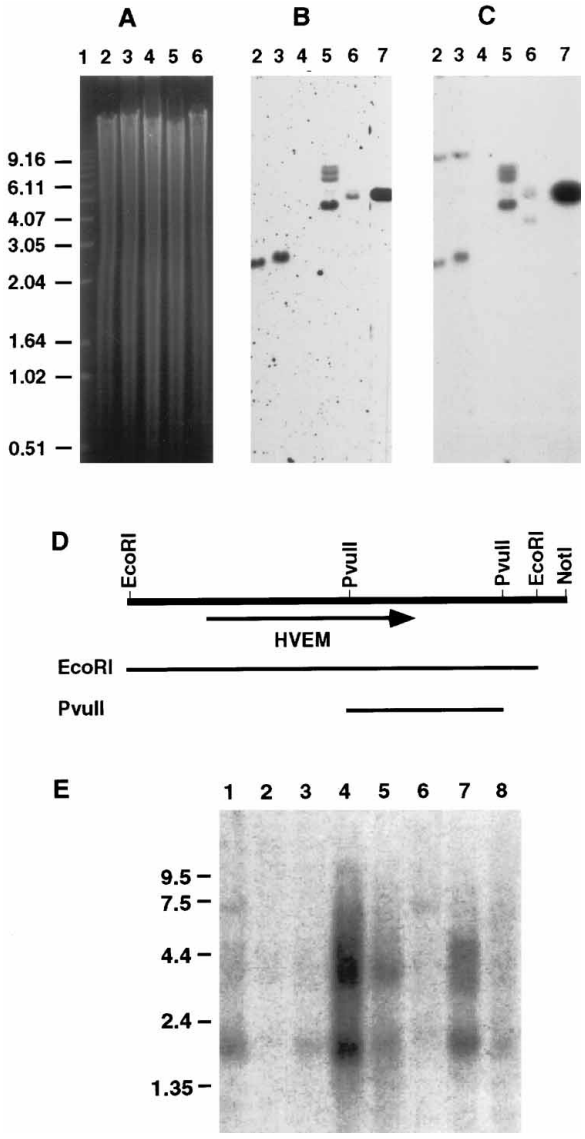

Figure 3. Southern and Northern Blots for Detection of HVEM–Homologous DNA and RNA in Cells and Tissues

(A–C) Cell DNAs were extracted and digested with BamHI and the fragments separated by electrophoresis and transferred to Duralon nylon membrane for hybridization. The DNAs were from HeLa (lanes 2), HEp-2 (lanes 3), CHO-K1 (lanes 4), CHO-HVEM12 (lanes 5), and Vero (lanes 6) cells. BamHI-digested plasmid, pBEC580, was also included (lanes 7).

(A) Photograph of the ethidium bromide–stained gel.

(B) Autoradiogram of the blot using the PvuII probe indicated in (D).

(C) Autoradiogram of the blot using the EcoRI probe indicated in (D).

(D) Schematic diagram of the HVEM cDNA and fragments used to generate probes. Numbers on the left of (A) and bands in lane 1 indicate molecular size markers (kbp).

(E) A Northern blot (Clontech) of polyadenylated RNAs extracted from various human tissues and hybridized with a ^{32}P-labeled probe from the PvuII fragment indicated in (D). The RNAs were extracted from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Amounts on the blot were normalized with respect to actin mRNA content.

Infection of HVEM–expressing cells (CHO-HVEM12 and ST-HVEM1) by KOS-gL86 was inhibited in a dose-dependent manner by anti–HVEM serum or HVEM:Fc but not by preimmune serum or normal rabbit IgG (Figures 5B and 5C). In fact, preimmune serum reproducibly enhanced infection. As expected, neither anti–HVEM serum nor HVEM:Fc inhibited PRV infection of ST-HVEM1 cells (data not shown).

To determine whether the anti–HVEM serum inhibited HSV binding, CHO-HVEM12 cells were exposed to radiolabeled KOS at several virus concentrations to quantitate virus binding at 4°C in the presence of anti–HVEM serum or preimmune serum at a 1:90 dilution. This dilution of antiserum completely inhibited HSV-1 infection at all input doses of virus tested (Figure 5B; data not shown) but did not inhibit virus binding (data not shown). Thus, the antibodies blocked infection by interfering with penetration (or possibly with events leading to uncoating and expression of the genome). The antibodies did not render the cells unresponsive to virus entry in general or inhibit cell functions required for gene expression. Antibody-treated ST-HVEM1 cells remained fully susceptible to PRV entry and expressed β-galactosidase from the viral genome.

The ability of HVEM to mediate entry of other strains of HSV was determined. CHO-IEβ8 cells transfected with HVEM–expressing or control plasmid were challenged with various concentrations of different HSV-1 and HSV-2 strains. These cells contain the Escherichia coli *lacZ* gene downstream of an HSV-1 immediate-early promoter and express β-galactosidase upon viral entry and delivery of the HSV trans-inducer VP16 (Campbell et al., 1984; Batterson and Roizman, 1983) into the cell. Expression of HVEM significantly enhanced entry of all wild-type HSV-1 and HSV-2 strains tested (Figure 6).

CHO-IEβ8 cells transfected with control plasmid, like parental CHO-K1 cells, did not completely resist entry of HSV-1(MP) and HSV-2(333) (Figure 6). Some hamster factor probably mediated their entry, albeit inefficiently. HSV-2 strains in general infect CHO-K1 cells more efficiently than do HSV-1 strains (Shieh et al., 1992). HSV-1(MP) and HSV-1(KOS)804 are syncytial mutants with enhanced ability to induce cell fusion due to missense mutations in gK (Pogue-Geile and Spear, 1987; Roop et al., 1993). HSV-1(ANG) also has a syncytial phenotype due to a missense mutation in gB (Weise et al., 1987). Thus, the syncytial phenotype does not explain the enhanced ability of HSV-1(MP) to infect CHO cells.

Expression of HVEM failed to enhance the entry of HSV-1(KOS)rid1 (KOS-rid1), HSV-1(KOS)rid 2 (KOS-rid2; data not shown), and HSV-1(ANG) (Figure 6). KOS-rid1 and KOS-rid2 are mutants selected for resistance to gD-mediated interference. Single amino acid substitutions in gD (Q27P or Q27R, respectively) account for the ability of these mutants to enter cells despite the block imposed by expression of wild-type gD by the cells (Dean et al., 1994). HSV-1(ANG) has the same amino acid substitution as KOS-rid2 (and other substitutions relative to KOS gD) and is also resistant to gD-mediated interference (Dean et al., 1994). KOS-rid1 and KOS-rid2 are somewhat impaired, compared with the parental strain KOS, in ability to infect human Hep-2 cells, but they infect HEp-2 cells much more efficiently than CHO-K1

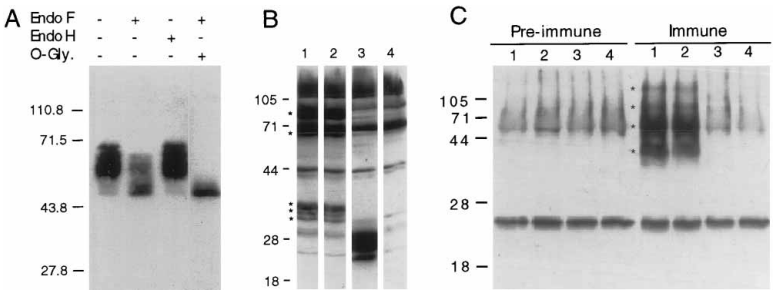

Figure 4. Expression of the HVEM:Fc Hybrid Protein and Epitope-Tagged HVEM-257Flu and Immunoprecipitation of HVEM-257Flu

(A) Samples of purified HVEM:Fc were appropriately treated for incubation with Endo F, Endo H, or Endo F/O-glycanase as indicated, subjected to SDS–polyacrylamide gel electrophoresis, and transferred to nitrocellulose for Western blot analysis with anti-rabbit IgG peroxidase conjugates and substrate for chemiluminescent detection.

(B and C) CHO-K1 cells were transfected with two different subclones of pBEC14 for expression of HVEM-257Flu (lanes 1 and 2), with pMN114 for expression of a Flu-tagged truncated version of HSV-1 gL (lane 3), or with pcDNA3, a control plasmid (lane 4). At 48 hr after transfection, cell lysates were prepared and either immediately added to sample buffer for SDS–polyacrylamide gel electrophoresis (B) or mixed with preimmune or immune rabbit serum for immunoprecipitation, followed by solubilization of the immune precipitates for SDS–polyacrylamide gel electrophoresis (C). After electrophoresis, the separated proteins were transferred to nitrocellulose for Western blotting with the anti-Flu MAb 12CA5. Asterisks indicate the multiple Flu-tagged forms of HVEM detected. Numbers at the left of each panel indicate molecular mass markers in kDa.

cells (Dean et al., 1994). We conclude that viruses carrying the mutant (or ANG) form of gD cannot use HVEM as a mediator of entry but enter human cells by other pathways.

HVEM–expressing CHO cells were permissive for viral replication (data not shown), indicating that the principal block to replication in CHO-K1 cells is at the stage of virus entry. CHO-HVEM12 cells infected with KOS or HSV-1(F) produced 10,000-fold or 100-fold more progeny virus, respectively, than did control CHO-C8 cells or CHO-K1 cells, but 10-fold to 100-fold less than did HeLa cells.

HVEM–Mediated Entry of HSV into Human Cells

Use of the rabbit antiserum to detect HVEM on surfaces of human cells revealed that activated peripheral blood mononuclear cells expressed higher levels of HVEM than cell lines such as 293 and HeLa (C. Ware, R. I. M., and P. G. S., unpublished data). Phytohemagglutinin-activated T cell blasts (greater than 95% CD3+ and CD25+) were prepared and shown to express HVEM (Figure 7A). These cells were exposed to viruses expressing wild-type gD (KOS-gL86) or a mutant form of gD (HSV-1[KOS]rid1-tk12 [KOS-rid1-tk12], a β-galactosidase–expressing version of KOS-rid1) in the absence or presence of anti–HVEM serum or preimmune serum. In absence of either serum, the T cell blasts were more susceptible to infection with KOS-gL86 than with KOS-rid1-tk12 (Figure 7B). As was noted with the HVEM–expressing CHO and ST cells, anti–HVEM serum had a dose-dependent inhibitory effect on infection and preimmune serum had a stimulatory effect, but only with KOS-gL86 (Figure 7B). Results similar to those shown in Figure 7 were obtained with activated T cell blasts from four different donors. We conclude that infection of T cell blasts with KOS-gL86 is largely HVEM–dependent.

Discussion

Demonstration that a new member of the TNF/NGF receptor family, HVEM, can mediate entry of HSV identifies another important family of cell-surface receptors used by viruses for entry. This adds to the growing body of evidence that multiple cell-surface components can be required for each entry event. Adenoviruses bind to cells via fibers extending from vertices of the icosahedral virions through as yet undefined interactions. Entry is then facilitated by interaction of a protein at the base of each fiber with cell-surface integrins (Wickham et al., 1993). Human immunodeficiency virus binds to cells via interaction of gp120 with CD4 (Dalgliesh et al., 1984; Klatzmann et al., 1984; Maddon et al., 1988), but entry requires cofactor activity which, for different virus strains, can be provided by various members of the chemokine receptor family (Feng et al., 1996; Deng et al., 1996; Dragic et al., 1996; Choe et al., 1996; Doranz et al., 1996; Alkhatib et al., 1996). Similarly, HSV binds to cell-surface GAGs, but entry requires mediator activity, which was shown here to be provided by HVEM. It should be noted that both GAGs and a mediator of entry such as HVEM are required for HSV infection. Cells lacking GAGs (Shieh et al., 1992; Banfield et al., 1995) or a mediator can be 100–1000 times more resistant to infection than cells expressing both.

The stage of HSV entry at which HVEM operates must be either the membrane fusion reaction that occurs after binding of virus to cell-surface GAGs or the release of internal virion proteins, including VP16, from sites of entry and their transport to the cell nucleus. This follows from our findings that virus binding to CHO-K1 cells occurs efficiently in the absence of HVEM or in the presence of anti–HVEM antibody. Also, the ability of HVEM to enhance HSV entry can be detected in CHO-IEβ8 cells, which carry a reporter gene under control of an immediate-early HSV-1 promoter. In these cells, introduction of input viral VP16 into the cell nucleus suffices to induce β-galactosidase expression and score an entry event.

It seems likely that HVEM interacts with one or more of the virion envelope glycoproteins to trigger membrane fusion or induce postfusion uncoating events. The finding that HVEM:Fc can inhibit HSV infection is consistent with direct interaction with virion surface proteins. Candidate proteins include the four envelope glycoproteins (gB, gD, gH, and gL) required for HSV-1 entry but not for binding of virus to cells. Functional interaction of virions with HVEM is influenced by gD because amino acid substitutions in gD eliminated ability of HSV-1 to

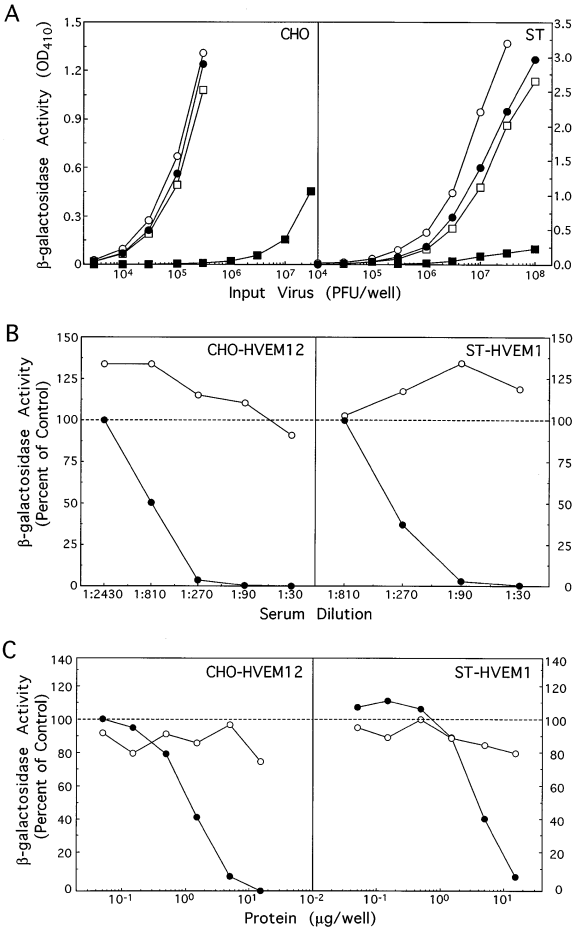

Figure 5. Enhanced Entry of HSV-1(KOS) into HVEM–Expressing Cell Lines and Inhibition of Infection by Anti–HVEM Antibodies or HVEM:Fc

(A) Several HVEM–expressing and control cell lines were obtained by transfection of CHO-K1 cells or ST cells with pBEC10 or control plasmid pcDNA3, followed by selection for stable maintenance of the plasmid. Representative clones were plated in 96 well plates and exposed to KOS-gL86 at the doses indicated. Later (6 hr), viral entry was quantitated as described in the legend to Figure 1. CHO-HVEM11 and ST-HVEM1 (open circles), CHO-HVEM9 and ST-HVEM22 (closed circles), and CHO-HVEM12 and ST-HVEM2 (open squares) were isolated after transfection with the HVEM–expressing plasmid pBEC10. CHO-C8 and ST-C8 (closed squares) were isolated after transfection with the control plasmid pcDNA3.

(B and C) CHO-HVEM12 cells or ST-HVEM1 cells were plated in 96 well dishes. In (B), the cells were exposed to preimmune or immune rabbit serum at the dilutions indicated for 30 min at 37°C. Various amounts of KOS-gL86 were then added in one-fifth volume, and incubation continued for 2 hr. In (C), virus was mixed with various concentrations of HVEM:Fc or normal rabbit IgG and incubated for 30 min at 37°C. The mixtures (50 μl) were added to washed cells, and incubation continued for 2 hr. The virus–serum or virus–HVEM:Fc mixtures were then removed and the cells exposed briefly to low pH buffer to inactivate residual extracellular virus. The cells were washed and incubated for an additional 4 hr before lysis and addition of ONPG. The amount of virus added was 10^7 pfu/well in (B) and 10^6 pfu/well in (C). Open symbols were preimmune serum (B) or normal rabbit IgG (C). Closed symbols were immune serum (B) or HVEM:Fc (C).

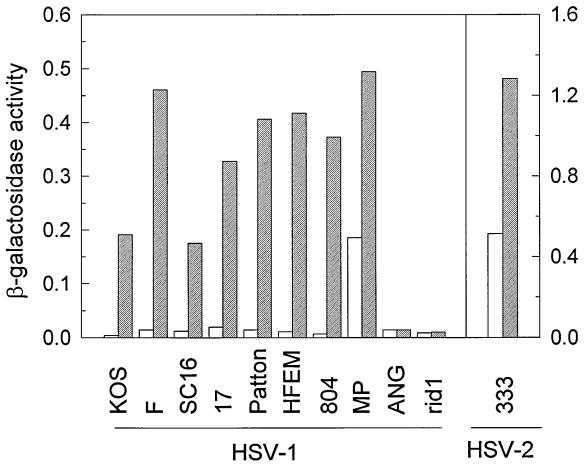

Figure 6. Enhanced Entry of HSV-1 and HSV-2 Strains into HVEM–Expressing CHO-IEβ8 Cells

CHO-IEβ8 cells were transfected with the HVEM–expressing plasmid pBEC10 (diagonally hatched bars) or the control plasmid pcDNA3 (open bars). At 24 hr after transfection, the cells were replated in 96 well dishes and, 24 hr later, exposed to various input doses of each of the virus strains indicated. Later (6 hr), the cells were lysed and ONPG added for the quantitation of viral entry. The results presented are for a single input dose of virus (30,000 pfu added per well) in the linear range of the dose response curve.

enter cells via HVEM (Figure 6) but not via other pathways of entry (Dean et al., 1994).

The role of HVEM in HSV entry could be completely dissociable from its normal physiological role. Ligand or ligands for HVEM and the consequences of ligand–receptor interaction remain to be identified. There is no obvious relationship between HSV envelope proteins and members of the TNF/NGF ligand family, although virions need not interact with HVEM in the same manner as its natural ligand. For members of the TNF/NGF receptor family, binding of ligand results in aggregation of receptors and activation of specific signal transduction pathways (Armitage, 1994; Heller and Krönke, 1994; Rothe et al., 1994). Binding of cognate receptors by members of the TNF or NGF family can have a variety of effects, including induction of cell proliferation, differentiation, or apoptosis, depending on the particular receptor, ligand, and other factors (Smith et al., 1994; Rabizadeh and Bredesen, 1994; Maness et al., 1994). Interestingly, another human herpesvirus, Epstein-Barr virus, expresses a membrane protein that interacts through a cytoplasmic domain with cytoplasmic proteins responsible for transmitting signals usually initiated by ligand binding to a TNF receptor family member (Mosialos et al., 1995). If any aspect of signaling through HVEM requires the C-terminal 26 amino acids, we can rule out its requirement for HSV entry based on the ability of HVEM-257Flu to mediate infection of CHO cells (data not shown).

Several lines of evidence indicate that other mediators of HSV entry have yet to be identified. First, although the cDNA for HVEM was isolated from a HeLa cell library, HVEM is probably not the principal mediator of HSV entry into HeLa cells. The anti–HVEM serum and HVEM:Fc had only marginal ability to block HSV-1 infection of HeLa cells (data not shown). Second, strains

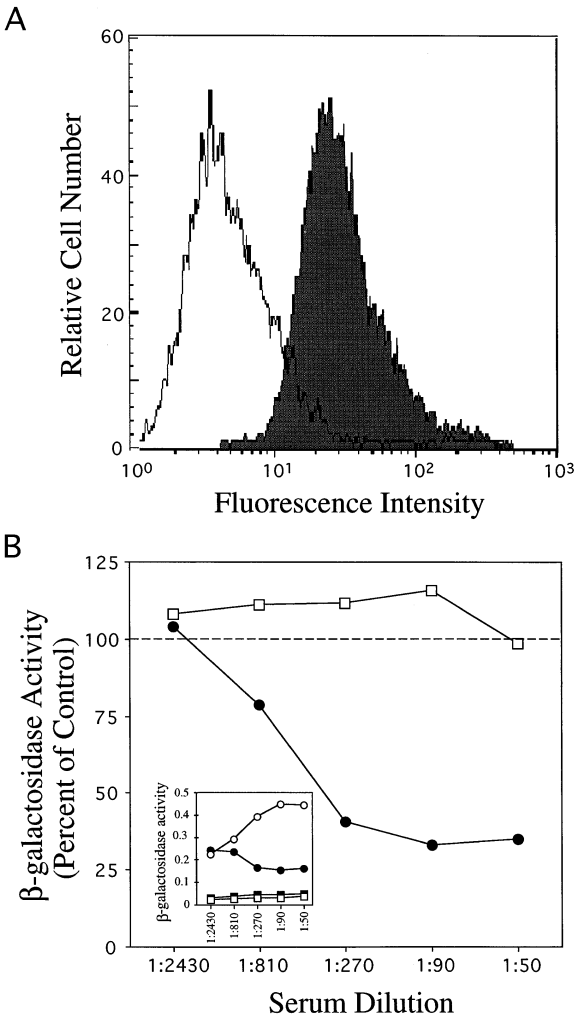

Figure 7. Expression of HVEM on Human T Lymphoblasts and HVEM–Dependent Infection by HSV-1 Expressing Wild-Type gD

(A) Activated T cells were incubated with anti–HVEM antiserum (shaded profile) or preimmune serum (open profile), followed by a fluoresceinated second antibody, and analyzed by flow cytometry. (B) Activated T cells were infected with KOS-gL86 or KOS-rid1-tk12 (10^7 pfu/well) in the absence or presence of anti–HVEM antiserum or preimmune serum. The cells were lysed and β-galactosidase activity quantitated at 6 hr after infection. The inset presents β-galactosidase activity expressed by KOS-gL86 (circles) or KOS-rid1-tk12 (squares) with preimmune (open symbols) or immune (closed symbols) serum. The large panel presents β-galactosidase in anti–HVEM–treated samples as a percentage of control values obtained with an equivalent concentration of preimmune serum, for KOS-gL86 (closed circles) and KOS-rid1-tk12 (open squares).

KOS-rid1, rid2, and HSV-1(ANG) can infect various human cell types but fail to infect HVEM–expressing CHO-K1 cells. Third, CHO-K1 cells express some factor that can mediate the entry of certain HSV strains, especially HSV-2 strains. Finally, continued screening of the HeLa cell cDNA library with other strains of HSV-1 indicates the presence of other genes that can mediate HSV entry (M. S. Warner, R. J. Geraghty, R. I. M., and P. G. S., unpublished data). These genes are not likely to encode basic fibroblast growth factor receptor, previously proposed to mediate HSV entry (Kaner et al., 1990), because its expression in resistant cells did not enhance susceptibility to HSV entry (Shieh and Spear, 1991; Mirda et al., 1992). Mannose-6-phosphate receptors have also been proposed to mediate entry of HSV-1, but cell mutants lacking these receptors remain fully susceptible to viral entry (Brunetti et al., 1995). The effect of expression of these receptors in cells resistant to infection needs to be explored.

The fact that HVEM enhances entry of the wild-type HSV-1 and HSV-2 strains tested and mediates HSV entry into activated T cells highlights its probable importance in HSV pathogenesis. Several reports have described the replication of HSV in activated T cells (Pelton et al., 1977; Rinaldo et al., 1978; Teute et al., 1983) and the presence of infected lymphocytes in biopsies of cutaneous lesions (Boddingius et al., 1987). Because HVEM is a principal mediator of HSV entry into activated T cells, and subtle genetic alterations in HSV can eliminate ability to utilize HVEM as an entry mediator, virus strains in the field may differ in ability to infect activated T cells. The virulence of a virus strain is influenced by its ability to infect lymphocytes, whether this infection promotes virus spread or abrogates localized immune responses. Discovery of HVEM opens new areas of investigation, including exploration of factors that induce HVEM expression in lymphocytes and other human cells, potential role of signal transduction in viral entry or viral replication, and effects of human or viral genetic polymorphisms on HVEM–virus interactions.

Experimental Procedures

Cells and Viruses

CHO-K1, HEp-2, HeLa, Vero, and ST cells were obtained from the American Type Culture Collection and HT1080 cells from Dr. N. Bouck (Northwestern University). The CHO-IEβ8 cell line was isolated after transfection of CHO-K1 cells with pMLP01, a plasmid having the E. coli *lacZ* gene under control of the HSV-1 ICP4 promoter and expressing β-galactosidase upon infection of cells with HSV (M. L. Parish, R. I. M., and P. G. S., unpublished data).

Wild-type virus strains used were HSV-1(KOS), HSV-1(HFEM), HSV-1(Patton), HSV-1(F) (Ejercito et al., 1968), HSV-1(SC16), HSV-1(17) (McGeoch et al., 1988), and HSV-2(333). Mutant strains included KOS-rid1 and rid2 (Dean et al., 1994), HSV-1(KOS)804 (Little and Schaffer, 1981), HSV-1(MP) (Hoggan and Roizman, 1959), and HSV-1(ANG) (Munk and Donner, 1963; Weise et al., 1987; Dean et al., 1994). KOS-rid1-tk12 is a recombinant virus produced by inserting the E. coli *lacZ* gene driven by the HSV-1 ICP4 promoter in place of the thymidine kinase gene of KOS-rid1. These strains were propagated by passage on HEp-2 cells and titered on Vero cells. KOS-gL86, a mutant in which the E. coli *lacZ* gene with CMV promoter replaces part of the gL open reading frame (M. J. Novotny and P. G. S., unpublished data), was propagated and titered on gL-expressing Vero cell transfectants. A PRV mutant, in which the gH gene was interrupted by the *lacZ* gene (Klupp et al., 1994), was obtained from T. C. Mettenleiter and propagated and titered on gH-expressing Vero cell transfectants. Mutant viruses obtained from these complementing cell lines were fully infectious for and expressed β-galactosidase in noncomplementing cells but produced only noninfectious virus.

Infectivity Assays

Infectivity assays were based on quantitation of β-galactosidase expressed from the viral genome or by the β-galactosidase–expressing cell line CHO-IEβ8. Adherent cells were plated in 96 well tissue culture dishes (2–4 × 10^4 cells/well) at least 16 hr prior to infection. Cells were washed and exposed to virus (in 50 μl of phosphate-buffered saline [PBS] containing glucose and 1% calf serum

[PBS-G-CS]) for 6 hr at 37°C before solubilization in 100 μl of PBS containing 0.5% NP-40 and the β-galactosidase substrate, o-nitrophenyl β-D-glucopyranoside (ONPG, 3 mg/ml). The reaction was monitored by spectrometry at several timepoints after the addition of ONPG to define the interval over which the generation of product was linear with time (Dynatech ELISA reader or a Spectromax 250). Alternatively, cells plated in 6 well tissue-culture dishes were exposed to virus and infected cells visualized using the β-galactosidase substrate X-gal (GIBCO Laboratories), which yields an insoluble blue reaction product. After infection, washed cells were fixed (PBS containing 2% formaldehyde and 0.2% gluteraldehyde), permeabilized (2 mM $MgCl_2$ containing 0.01% deoxycholate and 0.02% NP-40), and incubated with buffered X-gal (0.5 mg/ml). Antibody inhibition assays were performed as described for infectivity assays in 96 well plates, except that rabbit serum diluted in PBS-G-CS was added to the cells (50 μl/well) 30 min before addition of virus (10 μl/well). After 2 hr with the serum–virus mixtures, the cells were treated with 0.1 M citrate buffer (pH 3.0) for 1 min to inactivate extracellular virus (Huang and Wagner, 1964; Highlander et al., 1987). The cells were then washed and incubated in PBS-G-CS for 4 hr before solubilization and quantitation of β-galactosidase activity. Assays for inhibition of infection by HVEM:Fc were similar, except that dilutions of virus were mixed with HVEM:Fc or rabbit IgG and incubated for 30 min at 37°C prior to addition of the mixtures (50 μl/well) to washed cell monolayers.

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of freshly collected heparinized blood and incubated 3 days at 10^6 cells/ml in RPMI-1640 containing 10% fetal bovine serum and purified phytohemagglutin (Sigma Chemicals) at 5 μg/ml. T cell blasts were isolated on 50% Percoll cushions and were routinely more than 95% CD3+ and more than 98% CD25+. HVEM expression was determined by incubating cells with the anti–HVEM rabbit serum or preimmune serum, followed by goat anti-rabbit fluoresceinated secondary antibodies and flow cytometry (Beckton-Dickenson FACScan). Remaining cells were dispensed into 96 well plates (10^5 cells/well) for infectivity studies. Assays were as described above except that after 2 hr of incubation, the virus inocula were removed and replaced with medium containing preimmune or immune serum at the same concentrations as present during virus inoculation.

The binding of virus to cells was quantitated as described previously (Shieh et al., 1992) using ^{3}H-thymidine-labeled purified HSV-1(KOS).

Isolation and Sequencing of the HVEM cDNA

A unidirectional HeLa cell cDNA expression library cloned into pcDNA1 in E. coli (InVitrogen) was plated onto 100 150 mm Luria-Bertani plates containing appropriate drugs (1.5×10^5 bacteria/plate). Colonies were pooled by scraping and frozen as 100 glycerol stocks. Samples of each stock were combined into groups of 10 and grown to stationary phase in broth. Plasmids prepared from each culture were transfected into CHO-K1 cells using LipofectAMINE (GIBCO Laboratories; 1.5 μg of plasmid and 5 μl of LipofectAMINE/35 mm culture). For controls, cells were transfected with pMN84, a plasmid expressing β-galactosidase, or were incubated with LipofectAMINE alone. At 30 hr after transfection, the cells were washed, inoculated with KOS-gL86 at about 100 pfu per cell, and then stained with X-gal as described above. Transfection efficiencies ranged from 30%–55% of cells based on expression of β-galactosidase from pMN84 in uninfected cells. In the first round of screening the cDNA library, plasmids from one group of 10 bacterial stocks from the library converted about 20–30 cells in the monolayer to susceptibility to KOS-gL86 infection. The frequency of conversion to susceptibility was about 10 times higher for one of the 10 stocks in this group. This stock was divided again into 100 pools, and, by an iterative process, two bacterial clones were obtained that yielded plasmids (pBEC580 and pBEC748) with the desired phenotype.

Both strands of the cDNA insert of pBEC580 were sequenced using Sequenase (Amersham) and T7 and Sp6 primers, as well as other primers generated as sequence was obtained. All primers were obtained from the Northwestern University Biotechnology Center. Single-strand sequencing of pBEC748 revealed the insert to be the same as that of pBEC580.

Southern and Northern Blots

Genomic DNAs were isolated (Hirt, 1967), digested with BamHI, and electrophoresed on 0.8% agarose gels. Southerns were performed by standard procedures. Briefly, DNA was transferred to Duralon nylon membrane. Randomly primed probes were generated using digoxigen-labeled nucleotides (Genius Kit; Boehringer Mannheim) or ^{32}P-labeled nucleotides. Prehybridization was for 2 hr in hybridization buffer with Denhardt's reagent and 50% formamide. After hybridization with probe at 42°C for 24 hr, membranes were incubated twice for 15 min at 65°C with 0.2 × SSC containing 0.1% SDS. The blot was exposed to X-ray film at −70°C (for ^{32}P-labeled probes) or processed for digoxigen detection by chemiluminescence (Boehringer Mannheim), utilizing the alkaline phosphatase substrate CDP-STAR™ (Tropix).

Northern analysis was performed using a commercial blot of polyadenylated RNAs extracted from various human tissues (Clontech). Prehybridization and hybridization of the blot were done as described above at 42°C and washes with 0.2 × SSC containing 0.1% SDS were at 50°C. The blot was exposed to X-ray film at −70°C.

Plasmids and Stably Transfected Cell Lines

Plasmid pBEC10, carrying the HVEM insert and a neomycin-resistance gene, was generated by cloning a HindIII–XhoI fragment of pBEC580 into pcDNA3. pBEC14, expressing HVEM-257Flu, was generated in several steps. The HindIII-to-SfiI fragment of the HVEM insert from pBEC580 was modified by deletion between the BamHI and BstYI sites, then inserted between HindIII and EcoRI sites of pMN104 (blunt-end ligation between the SfiI and EcoRI sites after Klenow treatment). pMN104 contains an oligonucleotide, inserted between the EcoRI and XbaI sites of pcDNA3, that encodes 11 amino acids (EFYPYDVPDYASL) plus a stop codon, including a 9 amino acid Flu epitope (underlined, Wilson et al., 1984). pMN114, also derived from pMN104, expresses a truncated Flu-tagged version of HSV-1 gL (M. J. Novotny and P. G. S., unpublished data). pBL58, expressing a hybrid form of HVEM (the ectodomain fused to the hinge, C_H2 and C_H3 domains of the rabbit IgG heavy chain), was generated in several steps. It consists of a cytomegalovirus promoter from pcDNAneo (SpeI to HindIII); HindIII to XbaI from pGEM3; the ectodomain of HVEM from pBEC580 (NheI site to a PvuII site just downstream of the last Cys residue); a fragment of rabbit IgG heavy chain cDNA from plasmid 3-4 (obtained from K. Knight at Loyola University Medical Center) including an EcoRI site added by polymerase chain reaction 5′ to the rabbit sequence ACAAGACCGTTGC and extending to a PstI site downstream of the reading frame (after cleavage with EcoRI, the filled-in site was blunt end–ligated to the PvuII end of the HVEM fragment); and HindIII to NheI from pGEM4 (the PstI end of the rabbit sequence was blunt end–ligated to the HindIII site, and the NheI site was ligated to the SpeI site of the CMV promoter fragment).

Stable HVEM–expressing cell lines were produced by transfection of CHO-K1 cells and ST cells with pBEC10 and selection in medium containing Geneticin (500 μg/ml for CHO-K1 cells and 800 μg/ml for ST cells). Surviving cells were cloned by limiting dilution, and cell clones susceptible to KOS-gL86 infection were subcloned and expanded. Control cell lines were produced by transfecting CHO-K1 and ST cells with pcDNA3 and isolating Geneticin-resistant clones.

HVEM:Fc Production, Characterization, and Use as Immunogen

HVEM:Fc secreted into the medium of pBL58-transfected CHO-K1 cells was purified by Protein G–Sepharose chromatography. HVEM:Fc was denatured by boiling for 5 min in 0.5% SDS, 1% β-mercaptoethanol, and carbohydrate modifications determined by incubating overnight at 37°C with endo F (200 mU, 1% NP-40, 50 mM sodium phosphate [pH 7.5]) or endo H (1 mU, 50 mM sodium citrate [pH 5.5]) or without added enzyme (1% NP-40, 50 mM sodium phosphate [pH 7.5]). Alternatively, samples were digested overnight at 37°C with neuraminidase (4 mU, 50 mM sodium citrate [pH 4.5]), then denatured and incubated overnight at 37°C with endo F (200 mU) and O-glycosidase (0.5 mU) in 1% NP-40, 50 mM sodium phosphate (pH 7.5). Western blots of control and glycosidase-treated samples were probed with a mixture of anti-rabbit IgG peroxidase conjugates (GibcoBRL 9814SA and Sigma A6667) at concentrations

of 1:1000 in BLOTTO (10 mM Tris [pH 7.4], 150 mM NaCl, 5% powdered milk, 0.05% Tween-20), followed by chemiluminescent detection with ECL reagent and Hyperfilm-MP (Amersham).

Rabbit polyclonal antibodies were produced by subcutaneous injection of purified HVEM:Fc mixed with Hunter's TiterMax adjuvant at Pocono Farms, Inc. For immunoprecipitation, lysates were prepared with 1% Triton X-100 in 150 mM NaCl, 20 mM Tris–HCl (pH 8.0), containing protease inhibitors (2 μg/ml of aprotinin; 2 μg/ml of leupeptin; 1 μg/ml of pepstatin A; 5 mM phenylmethylsulfonyl fluoride) and mixed on ice with rabbit preimmune or immune serum (10 μl/200 μl of lysate). Samples of cell lysates and immunoprecipitates collected on Protein A–agarose were subjected to SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated in BLOTTO for 1 hr of blocking and then with the anti-hemagglutinin antibody 12CA5 (Wilson et al., 1984) diluted 1:5000 in BLOTTO, followed by horseradish peroxidase–coupled goat anti-mouse IgG (Boehringer Mannheim) diluted 1:10,000. Detection of second antibody was by incubation in ECL reagent and exposure to Amersham Hyperfilm-MP (Amersham).

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