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Functional Characterization of the HveA Homolog Specified by African Green Monkey Kidney Cells with a Herpes Simplex Virus Expressing the Green Fluorescence Protein

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We cloned the gene specified by African monkey kidney cells (Vero) that codes for the homolog of the herpes virus entry mediator (HveA) specified by HeLa cells. The primary sequence of the monkey HveA (HveAs) differed significantly from HveA. Single amino acid differences were distributed throughout the amino and carboxyl terminal portions of the HveAs in comparison with the HveA, whereas certain regions were highly conserved. The predicted membrane spanning domains of the two receptors differed substantially due to insertions and deletions of short amino acid sequences. The ability of HveAs to mediate HSV virus entry was tested in a series of experiments using the recombinant virus KOS/EGFP, which constitutively expressed the enhanced green fluorescence protein (EGFP) and Chinese hamster ovary cells (CHO) transformed with the HveAs gene. The KOS/EGFP virus was constructed by inserting an EGFP gene cassette within the intergenic region between the UL53 (gK) and UL54 (ICP27) genes. The KOS/EGFP virus formed viral plaques and replicated as well as the wild-type KOS virus. HveAs-transformed CHO cells constitutively expressing HveAs mediated herpesvirus entry efficiently, whereas cells transformed with the HveAs gene in the noncoding orientation did not mediate virus entry. A genetically engineered protein composed of the amino-terminal portion of the HveAs protein fused to the heavy chain of mouse IgG immunoglobulin as well as mouse antibodies raised against HveAs blocked virus entry into HveAs-transformed CHO cells. Thus, HveAs is the functional homolog of HveA.

INTRODUCTION

Early steps during productive infection of eukaryotic cells by herpes simplex virus include initial binding of virions to cell surfaces mediated by binding of viral glycoproteins gB and gC onto glycosaminoglycan chains that are ubiquitous on most cell surfaces (Spear, 1993). Virion penetration into cells occurs via the pH-independent fusion of the viral envelope with the cell membrane or an early endosome (Wittels and Spear, 1991) and is mediated by at least viral glycoproteins 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).

A gD receptor for virus entry, HveA, is an additional member of the tumor necrosis factor receptor family (Montgomery *et al.,* 1996; Whitbeck *et al.,* 1997; Geraghty *et al.,* 1998). HveA is expressed in various tissues, including liver, lung, kidney, spleen, and peripheral leucocytes, and it is the principal receptor for entry into human lymphoid cells but not into other cell types (Montgomery *et al.,* 1996). The cytoplasmic region of HveA binds to several members of the tumor necrosis factor-associated factor (TRAF) family, such as TRAF1, TRAF2, TRAF3, and TRAF5 but not to TRAF6. Transient transfection of HVEM (HveA) into human 293 cells causes activation of nuclear factor-kB (NF-kB), a transcriptional regulator of multiple immunomodulatory and inflammatory genes as well as activation of the Jun-containing transcription factor AP-1, a regulator of cellular stress-response genes. These results suggest that HveA is associated with signal transduction pathways that activate the immune response (Marsters *et al.,* 1997).

A second receptor for virus entry, HveB, is the poliovirus receptor-related protein 2 (Prr2). HveB mediates HSV-2 but not wild-type HSV-1 viral entry (Eberle *et al.,* 1995). Apparently certain mutants of HSV-1 use HveB for viral entry, and HveB but not HveA mediates pseudorabies virus entry. A third receptor, HveC, is the poliovirus receptor-related protein 1 (Prr1), which is a human member of the immunoglobulin superfamily (Lopez *et al.,* 1995). HveC mediates entry of several alphaherpesviruses, including HSV-1 and -2, pseudorabies virus (PRV), and bovine herpesvirus-1 (BHV-1). HveC is expressed in human cells of epithelial and neuronal origin and is the primary candidate for the coreceptor that mediates HSV-1 and -2 viral entry into epithelial cells on mucosa surfaces and virus spread to cells of the nervous system (Geraghty *et al.,* 1998). A fourth receptor, HveD, is the poliovirus receptor Pvr (Mendelsohn *et al.,* 1989). HveD mediates the entry of PRV and BHV-1 but not of HSV-1 (Geraghty *et al.,* 1998).

African green monkey kidney (Vero) cells are commonly used for the propagation of HSV-1 in many her-

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pesvirus laboratories as well as for the determination of HSV-1 virus entry and egress. Therefore we cloned, sequenced, and characterized the Vero HveA homolog, designated here as HveAs. Functional characterization of HveAs for HSV-1 virus entry was accomplished through the construction and use of a recombinant virus constitutively expressing the enhanced green fluorescence protein (EGFP) while retaining wild-type KOS-like characteristics. Our results indicate that HveAs is the functional homolog of HveA.

RESULTS

Construction and genetic characterization of the recombinant virus HSV-1 (KOS)/EGFP

Recently, we constructed the recombinant virus ΔgK/EGFP expressing an engineered enhanced green fluorescent protein (EGFP) from a gene cassette inserted in place of the glycoprotein K (gK) gene (Foster *et al.,* 1999). To facilitate our studies on HSV-1 entry, we engineered the virus KOS/EGFP constitutively expressing EGFP by inserting the HCMV-EGFP gene cassette into the intergenic region between the UL53 (gK) and the UL54 (ICP27) genes. First, plasmid pTF9202 was constructed to contain the HCMV-EGFP cassette in the intergenic region between the UL53 and UL54 genes. Next, the deleted ICP27-UL54 region of the d27–1 viral genome was rescued by plasmid pTF9202, while simultaneously transferring the CMV-EGFP gene cassette into the viral genome (Fig. 1). Similar methodologies were used to construct the ΔgK virus (Jayachandra *et al.,* 1997) and the ΔgK/EGFP virus (Foster *et al.,* 1999). Virus stocks from marker-rescue experiments were collected, individual virus plaques were plaque purified five times, and virus isolates were tested by diagnostic PCR. Primer gKTr2 is located within the deleted portion of the UL54 gene (Fig. 1c). Amplification with this primer is possible only if the UL54 gene has been rescued by the pTF9202 plasmid. Complementary DNA sequences to primer gKTr2 are present in plasmid pTF9202 immediately upstream and downstream of the CMV/EGFP gene cassette. (Figs. 1c and 1d). Therefore amplification with primer pair UL52KpnI2/gKTr2 generated two DNA fragments of 1569 and 3261 bp against KOS/EGFP viral DNA (Fig. 1e, lane 3), while only the 1569-bp DNA fragment was generated against KOS viral DNA (Fig. 1e, lane 2). Primer pair UL52KpnI2/CMVBamHI detected the presence of the EGFP gene cassette within the KOS/EGFP viral genome as evidenced by the PCR-amplified DNA fragment of 2401 bp (Fig. 1e, lane 5), whereas it did not produce a PCR product against the KOS viral genome (Fig. 1e, lane 4). Additional PCR testing was performed to ensure that the KOS/EGFP virus was not contaminated with the d27–1 virus using primer pair UL52KpnI2/d27–1α (Fig. 1b). This primer pair produced the predicted size DNA fragments of 3241 and 1613 bp against KOS and d27–1 viruses (Fig. 1e, lanes 6, and 8, respectively), and the predicted DNA fragment of 4889 bp against the KOS/EGFP virus (Fig. 1e, lane 7). A second DNA fragment of 650 bp was of nonspecific origin.

Plaque phenotype, EGFP expression, and growth characteristics of the KOS/EGFP virus

The KOS/EGFP virus produced viral plaques similar in appearance to those produced by the wild-type virus KOS (Figs. 2a and 2c). Bright green fluorescence was detected in all infected cells when viral plaques were examined under the fluorescent microscope (Fig. 2b). Fluorescence from Vero cells infected with either KOS or KOS/EGFP viruses at an m.o.i. of 5 was detected using FACS at 24 h p.i. Nearly, all KOS/EGFP-infected cells emitted bright fluorescence, whereas KOS-infected cells did not emit any fluorescence (Fig. 2d).

To determine the replication characteristics of the KOS/EGFP virus, Vero cell monolayers were infected in triplicates with either KOS or KOS/EGFP virus at a m.o.i. of 10, and virus stocks were prepared at 12, 24, 36, and 48 h p.i. The KOS/EGFP virus replicated to high titers approaching those of the KOS parental virus (Table 1).

Isolation of the gene coding for the Vero HveA homolog (HveAs) and comparison of the HveA and HveAs predicted amino acid sequences

Primers specific for the HveA gene were used to isolate and clone the HveAs cDNA specified by Vero cells. Translation of the largest open reading frame of the HveAs cDNA sequence produced a putative protein of 283 amino acids sharing a high degree of homology with the previously described HveA receptor gene (Montgomery *et al.,* 1996). On the basis of the homology to HveA, this protein was designated the HveA simian homolog (HveAs). A comparison of the predicted amino acid sequences of HveA and HveAs is shown in Fig. 3. The two proteins were of equal length (283 amino acids) but contained a number of amino acid differences including (i) 41 amino acid substitutions of which 24 were to similar amino acids and 17 were to dissimilar amino acids. And (ii) amino acid deletions and insertions principally located within the intramembrane sequences. Specifically, HveAs contained a single amino acid insertion (aa 187 of HveAs) and a single amino acid deletion (aa 212 of HveA) and a segment of deleted amino acids (aa 216–218 of HveA) and inserted amino acids (aa 216 and aa 222–226 of HveAs). Characteristically, the carboxyl terminus of HveA contained three extra amino acids (P-N-H) in comparison with HveAs.

Functional characterization of the HveAs receptor

The ability of HveAs to mediate HSV-1 virus entry was assessed through the use of HveAs-transformed CHO cells, anti-HveAs antibody, and soluble HveA protein.

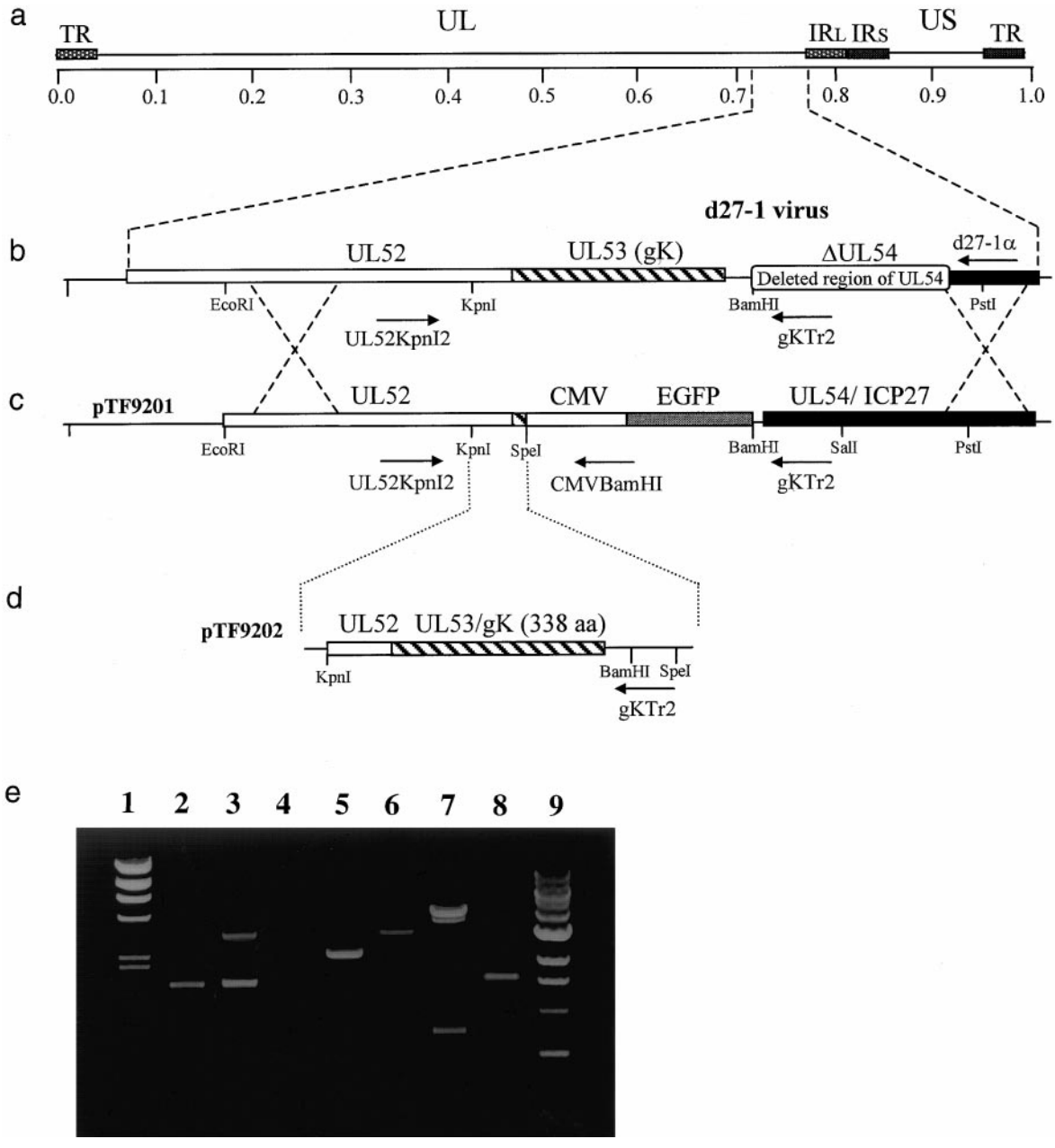

FIG. 1. Construction of recombinant KOS and ΔgK viruses constitutively expressing the EGFP gene. (a) The top line represents the prototypic arrangement of the HSV-1 genome indicating approximate map units. (b) The region of the mutant virus HSV-1 d27–1 genome containing the UL52, UL53, and the partially deleted UL54 genes with relevant restriction endonuclease sites. (c) Plasmid pTF9201, which contains the CMV-IE-EGFP gene cassette in place of the gK gene (Foster *et al.,* 1999). (d) Plasmid pTF9202 was produced by inserting the gK gene within the unique *Kpn*I/*Spe*I sites of plasmid pTF9201. The approximate location of PCR primers, UL52KpnI2, gKTr2, and CMVBamHI are shown (b–d). (e) Diagnostic PCR of plaque purified KOS/EGFP recombinant virus. DNA PCR products with the UL52KpnI2/gKTr2 primer pair (lanes 2 and 3) or the UL52KpnI2/CMVBamHI primer pair (lanes 4 and 5) used to detect the EGFP gene. Lanes 6–8: PCR diagnostic with primer pair UL52KpnI2/d27–1α used to determine the purity of viral stocks. Lanes 2, 4, and 6: KOS viral DNA. Lanes 3, 5, and 7: KOS/EGFP viral DNA. Lane 8: d27–1 viral DNA. Lane 1: lambda phage DNA digested with *Hin*dIII (marker). Lane 9: 1-kb molecular weight ladder.

Anti-HveAs serum was produced by genetic immunization using plasmid pCMV/HveAs, and it was tested for its ability to react with HveAs expressed in *Escherichia coli* as a fusion protein with the maltose-binding protein (MBP). Anti-HveAs serum reacted with a protein species migrating with an apparent molecular mass of 70 kDa in agreement with the expected apparent molecular mass of electrophoretically separated MBP/HveAs fusion protein (Fig. 4a, lane 2 of immune samples). Anti-HveAs serum did not react against MBP expressed in *E. coli* (Fig. 4a, lane 1 of immune samples). Similarly, pre-immune sera did not react with either MBP or MBP/HveAs proteins (Fig. 4a, lanes 1 and 2, respectively, of pre-immune samples).

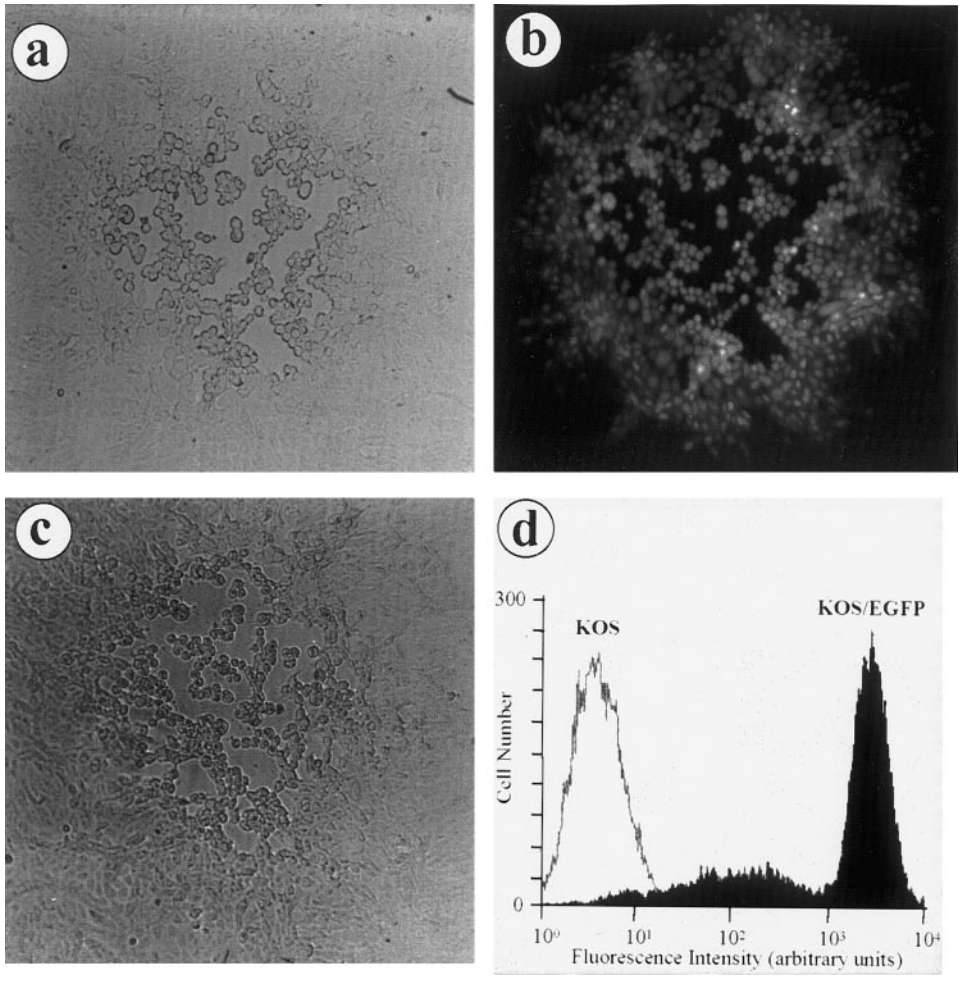

FIG. 2. Morphology and fluorescence detection of KOS/EGFP viral plaques. Vero cells were infected with either KOS (c) or KOS/EGFP virus (a and b) at an m.o.i. of 0.001 in 24-well plastic tissue culture plates. Individual viral plaques were photographed through the use of phase-contrast (a and c) and epi-fluorescence (b) microscope under live conditions at 48 h p.i. FACS analysis of KOS- and KOS/EGFP-infected cells (m.o.i. of 5) was performed at 48 h p.i. (d) as described previously (Foster *et al.,* 1999).

Next, CHO cells were transformed with either pCMV/HveAs or pCMV/sAevH. Plasmid pCMV/sAevH contains the HveAs gene in the opposite (noncoding) orientation. HveAs expression by CHO/HveAs and Vero cells was assessed using anti-HveAs serum and FACS analysis.

TABLE 1

Viral yields of KOS and KOS/EGFP viruses

	Titer (PFU/ml)			
Virus	12 h p.i.	24 h p.i.	36 h p.i.	48 h p.i.
KOS	5.0×10^7	1.5×10^8	2.0×10^8	2.0×10^8
KOS-EGFP	3.0×10^7	1.0×10^8	1.4×10^8	1.5×10^8

Note. Subconfluent Vero cell monolayers (approximately 8×10^5 cells) were infected with each virus at an MOI of 5, and at 12, 24, 36, and 48 h.p.i. the total number of infectious virions was determined. Viral yields represent one of three experiments in which individual numbers varied by less than 2-fold.

Anti-HveAs serum reacted strongly with paraformaldehyde-fixed Vero cells and CHO/HveAs cells (Figs. 4b and 4c, respectively, solid histograms). No background fluorescence was observed in control experiments in which Vero cells were incubated with pre-immune sera prior to incubation with goat-anti-mouse FITC-conjugate (Fig. 4b, unfilled histogram). Anti-HveAs antibody did not react with CHO/sAevH cells (Fig. 4c, unfilled histogram).

To facilitate virus entry blocking experiments, soluble HveAs protein was produced by first, constructing a gene cassette coding for the extracellular portion of HveAs fused to the Fc portion of mouse IgG. Next CHO cells were transformed with plasmid pCR/HveAs:Fc capable of constitutively expressing HveAs:Fc protein, and HveAs:Fc protein was purified from supernatant fluids of these cells using protein A sepharose beads (see Materials and Methods). The authenticity of HveAs:Fc protein was tested in immunoblots using anti-mouse-IgG antibody. A protein species with an apparent molecular

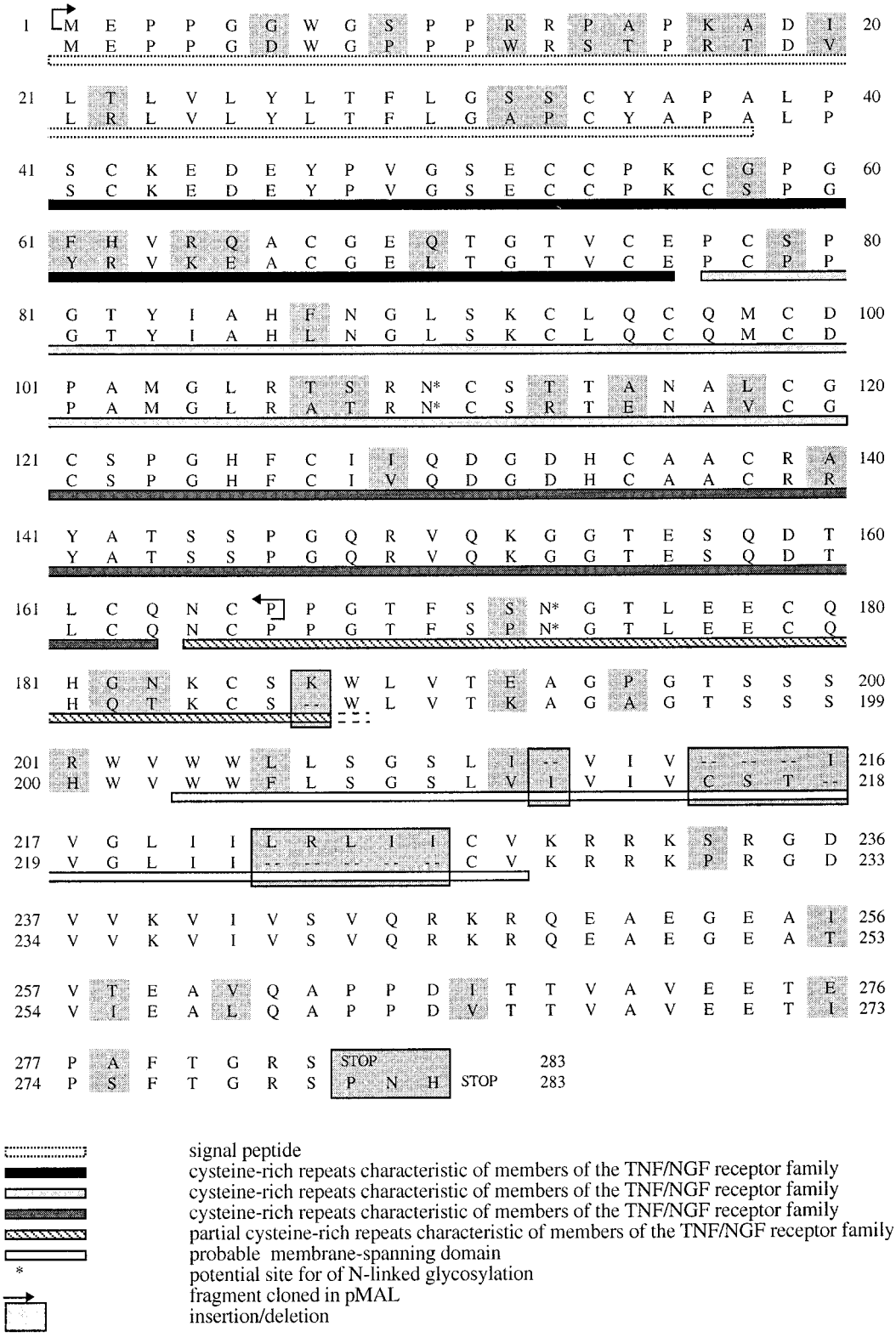

FIG. 3. Comparison of the amino acid sequences of the HveAs and HveAh. The HveAs (top lines) and HveA (bottom lines) amino acid sequences were aligned through the use of computer-assisted algorithms and visual inspection. Both proteins are 283 amino acids long. Relevant structural features of both proteins are marked as indicated.

mass of ~60 kDa was detected in immunoblots of electrophoretically separated HveAs:Fc protein (Fig. 5, lane 2), whereas anti-mouse IgG antibody did not react with supernatants from CHO/sAevH control cells (Fig. 5, lane 1). The HveAs:Fc-purified protein was sensitive to digestion by PNGase F, indicating that it contained *N*-glycosy-

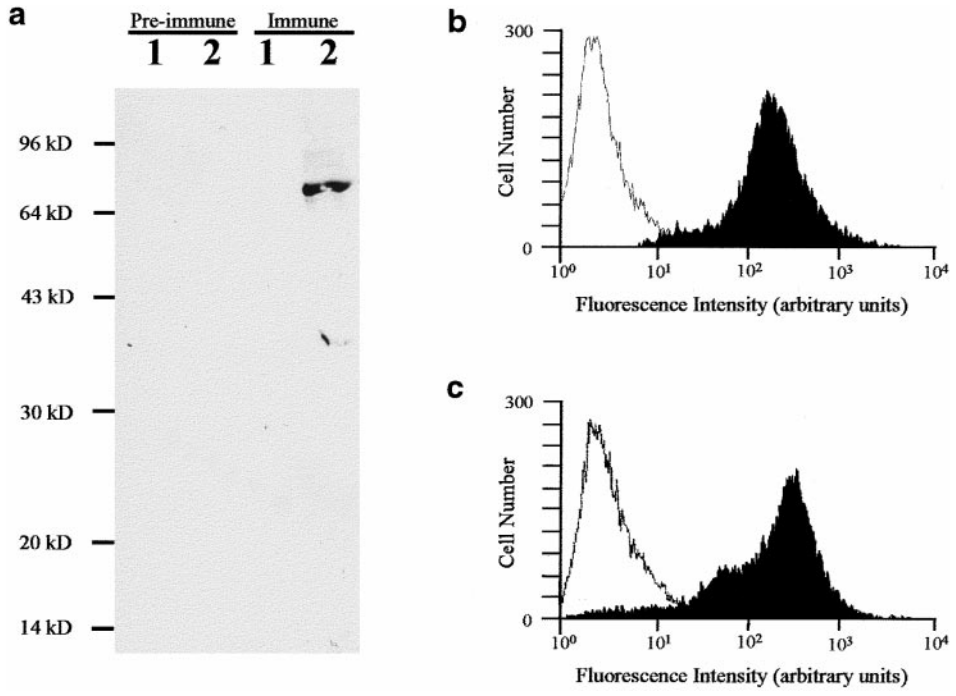

FIG. 4. Detection of HveAs expression in *E. coli*, Vero, and HveAs-transformed CHO cells using anti-HveAs serum. (a) Immunoblot of electrophoretically separated HveAs/MBP fusion protein after expression in *E. coli* using anti-HveAs serum produced by genetic immunization. Pre-immune and immune sera were tested as shown. Lanes labeled 1: MBP control. Lanes labeled 2: MBP/HveAs fusion protein. (b) Vero cells were analyzed by FACS after reaction with either anti-HveAs antibody followed by reaction with anti-mouse-IgG labeled with fluorescein (filled histogram) or with pre-immune sera as primary antibody (unfilled histogram). (c) CHO/HveAs (filled histograms)- and CHO/sAevH (unfilled histograms)-transformed cells were analyzed by FACS to detect HveAs using anti-HveAs antibody.

lated carbohydrates (not shown). A glycosylated protein with similar apparent mass was produced by a HveA:Fc fusion protein (Montgomery *et al.,* 1996)

Virus-entry experiments were performed by monitoring EGFP expression specified by KOS/EGFP virus. KOS/EGFP infection (m.o.i. of 5) of CHO/HveAs cells resulted in most cells emitting green fluorescence at 24 h p.i., indicating successful viral entry (Fig. 6A). In contrast, practically none of the KOS/EGFP-infected CHO/sAevH control cells emitted fluorescence (Fig. 6B). Next experiments were performed using the soluble HveAs:Fc protein. Pre-incubation of KOS/EGFP virions with supernatant fluids from CHO/sAevH control cells did not reduce the number of fluorescent cells, whereas pre-incubation with purified HveAs:Fc protein reduced the number of fluorescent cells substantially (Figs. 6C and 6D, respectively). Similarly, treatment of the CHO/HveAs cells with pre-immune control serum prior to infection with KOS/EGFP virus did not reduce the number of fluorescent cells, whereas treatment with anti-HveAs antibody serum caused a substantial decrease in the number of fluorescent cells (Figs. 6E and 6F, respectively).

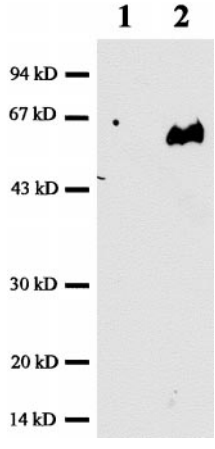

FIG. 5. Immunoblot detection of HveA:Fc expression from CHO/HveAs:Fc-transformed cells. Supernatants from either HveAs:Fc transformed CHO cells or naive CHO/sAevH control cells were subjected to protein A sepharose purification and prepared for immunoblot analysis. Immunoblots were reacted with goat-anti-mouse HRP reagent. Lane 1: reaction with supernatant concentrate from CHO cells. Lane 2: reaction with supernatant concentrate from HveAs:Fc-transformed CHO cells.

DISCUSSION

We report here the functional characterization of the herpes simplex HveA receptor homolog specified by African green monkey kidney cells, designated here as HveAs. These studies were undertaken because Vero cells constitute our primary cells in which virus entry and egress are studied. Investigation of the ability of HveAs to mediate HSV-1 virus entry was achieved through the

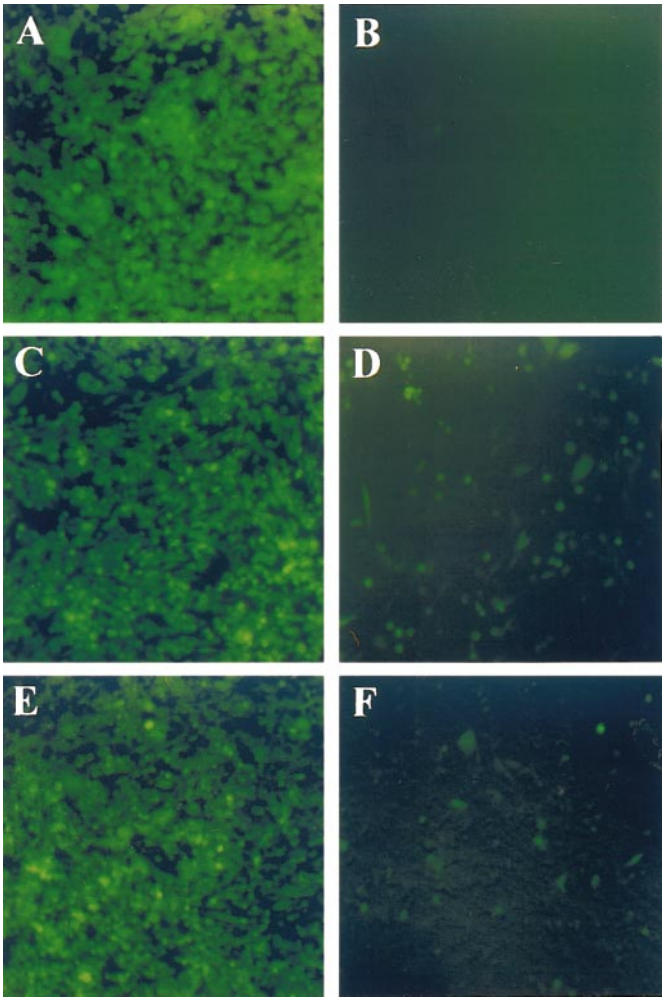

FIG. 6. Blocking of virus entry by HveAs:Fc soluble protein and by anti-HveAs antibodies. (A and C–F) CHO/HveAs cells infected with KOS/EGFP virus at an m.o.i. of 10. (A) Untreated control. (B) CHO/sAevH control cells (transformed with the HveAs gene in the noncoding orientation). (C and D) CHO/HveAs cells were pre-treated with pre-immune or anti-HveAs serum, respectively, for 30 min at room temperature prior to infection. (E and F) KOS/EGFP virus was pre-treated for 30 min at room temperature with CHO/sAevH control supernates and soluble HveAs:Fc, respectively, for 30 min at 4°C prior to infections.

use of a novel wild-type KOS-like herpesvirus constitutively expressing the EGFP protein immediately after viral entry into cells.

The KOS/EGFP virus

We inserted the EGFP gene cassette within the intergenic region between the UL53 and UL54 genes immediately after the poly-A sequence of the UL53 gene. The purpose of this construction was to enable the monitoring of viral entry through the detection of EGFP fluorescence. In addition, EGFP could serve as a fluorescent marker for the isolation of recombinant viruses containing gK mutations by simultaneously transferring mutated gK genes and the EGFP gene cassette to viral genomes. EGFP was selected because this protein is capable of emitting bright green fluorescence within cells in cell culture under physiological conditions without the requirement of exogenous substances. In addition, EGFP is highly stable under varying pH conditions, detergents and reducing agents enabling the direct measurement of EGFP accumulated expression (Chalfie *et al.,* 1994; Chalfie 1995). In this regard, EGFP detection provides a useful alternative to β-galactosidase and other enzymes because it does not require addition of toxic reagents that inactivate cells and viruses.

The growth characteristics and plaque morphology of the resultant KOS/EGFP virus were similar to wild-type KOS virus, indicating that insertion of the EGFP gene cassette between the two essential genes, UL52 and UL54 as well as expression of the EGFP proteins did not adversely affect virus replication. High levels of EGFP expression were achieved in KOS/EGFP virus-infected cells that was easily detected by fluorescence microscopy and quantified by FACS analysis. KOS/EGFP fluorescence was detected through the use of a fluorescent microscope as early as 5–6 h p.i. and fluorescent plaques were readily visualized at 12 h p.i. Fluorescence emitted from cells located within the center of each plaque increased with time, whereas newly recruited cells appeared weakly fluorescent until EGFP accumulated at later times. This radial feature of fluorescence emission enables an improved, time-dependent visualization of KOS/EGFP plaque formation in comparison with phase-contrast microscopy.

HveAs mediates HSV entry

The functionality of the HveA receptor was demonstrated principally by showing that CHO/HveA-transformed cells facilitated HSV-1 entry and that HSV-1 entry into these cells was blocked by either soluble HveA:Fc protein or anti-HveA antibodies (Montgomery *et al.,* 1996). We performed similar experiments with the exception that viral entry into cells was monitored through fluorescence detection of the EGFP protein specified by the KOS/EGFP virus. Collectively, these experiments showed that HveAs mediates HSV virus entry in a specific manner. Undiluted amounts of anti-HveAs antibody and CHO cell supernatants containing soluble HveAs:Fc protein reduced viral entry into vero cells by ~40 and 20%, respectively, as measured by FACS (not shown). The inability of anti-HveAs antibody and soluble HveAs:Fc to fully inhibit virus entry into Vero cells is due to either the use of the blocking reagents at nonsaturating conditions or the presence of the HveC and other receptors on Vero cell surfaces (Geraghty *et al.,* 1998).

The HveAs protein

Comparison of the cDNA-predicted amino acid sequences of HveA and HveAs proteins revealed conser-

vation of all cysteine residues with the exception of a single cysteine missing in the membrane-spanning portion of HveAs with respect to HveA. Furthermore overall structural features, which identified the HveA protein as a homolog to the TNF family of proteins, were conserved (Montgomery *et al.,* 1996). However, there was a significant divergence at the amino acid level between the HveA and HveAs proteins. Most amino acid substitutions were concentrated within specific regions of the amino terminus of the molecule and were to similar amino acids that are not expected to drastically alter the secondary structure of the molecule. However, certain amino acid substitutions were to dissimilar amino acids, which may alter structural properties of the protein. These were: S9 to P9, R12 to W12, P14 to S14, T22 to R22, S33 to P33, Q70 to L70, S79 to P79, T113 to R113, A115 to E115, A140 to R140, S172 to P172, G182 to Q182, and N183 to T183 in the extracellular portions of the proteins and S233 to P230, I256 to T253, T258 to I254, and E276 to I273 in the cytoplasmic portions of the proteins. Significant insertions and deletions were noted in the predicted intramembranous sequences of the HveAs and HveA proteins including a deletion that eliminated one cysteine residue in the HveAs sequence in comparison with the HveA sequence. However, both intramembranous sequences exhibited similar hydrophobic potential, indicating that both proteins should be effectively anchored into membranes. The carboxyl terminus of HveA ended in amino acids P-N-H, which were not present in the carboxyl terminus of the HveAs protein. Deletion of the terminal 26 amino acids of the HveA protein did not adversely affect the ability of this protein to mediate herpes simplex virus entry (Montgomery *et al.,* 1996); therefore the carboxyl terminal differences between HveAs and HveA are not expected to significantly alter the ability of HveAs to mediate HSV virus entry in comparison with HveA.

The primary sequences of gD specified by HSV and simian herpes B virus (SHBV) are 58% identical. However, regions of gD that are important for HSV-1 virus entry into Vero cells are highly conserved in HSV-1, HSV-2, and SHBV (Bennett *et al.,* 1992; Chiang *et al.,* 1994). Furthermore anti-HSV-1 gD monoclonal antibodies that block HSV-1 binding to soluble HveA recognize epitopes that overlap or are adjacent to these conserved regions (Nicola *et al.,* 1998). HSV enters into both HveA and HveAs-transformed cells efficiently, suggesting that conserved domains in the extracellular portions of HveA and HveAs proteins may serve as common functional sites for binding gD during viral entry.

MATERIALS AND METHODS

Cells and viruses

African green monkey kidney (Vero) cells were obtained from ATCC (Rockville, MD). Cells were propagated and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing sodium bicarbonate and 15 mM HEPES, and supplemented with 7% heat inactivated fetal bovine serum (FBS). V27 cells carry a stably integrated copy of the HSV-1 (KOS) ICP27 gene and were kindly provided by Dr. D. M. Knipe, Harvard Medical School. These cells were cultured in DMEM supplemented with 7% FBS (Rice and Knipe, 1990). HSV-1(KOS), the parental wild-type strain used in this study, was originally obtained from Dr. P. A. Schaffer (Dana-Faber Cancer Institute, Boston, MA). HSV-1(KOS) d27–1, which has a 1.6-kb *Bam*HI-*Stu*I deletion of the ICP27 gene, also was kindly provided by Dr. D. M. Knipe and was propagated in V27 cells (Rice and Knipe, 1990).

Reagents

Restriction and DNA modification enzymes were obtained from New England BioLabs (Beverly, MA). RNase and Proteinase K were purchased from Boehringer Mannheim (Indianapolis, IN). Gel fragment purification matrix and buffers (Prep-a-gene) were obtained from BioRad (Hercules, CA). Sequencing grade [^{35}S] dATP was obtained from DuPont/NEN (Wilmington, DE). Amplitaq, rTth XL Polymerase, and deoxynucleotide triphosphates were purchased from Perkin–Elmer (Foster City, CA). All synthetic oligonucleotide primers were synthesized by the LSU Gene Probes and Expression Systems Laboratory "GeneLab" using phosphoamidite chemistry on an Applied Biosystems ABI394 DNA/RNA synthesizer with Perkin–Elmer (Foster City, CA) reagents.

Isolation of the HveAs gene and construction of plasmids

Total RNA was extracted from Vero cells using TRIReagent (Molecular Research Center, Inc., Cincinnati, OH). A cDNA library was constructed using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech Inc., Uppsala, Sweden). All PCR amplifications were performed using the Gene-Amp PCR system 9600, AmpliTaq, and other Perkin–Elmer reagents (Perkin–Elmer, Norwalk, CT). PCR products were derived using HveA-specific primers (Montgomery *et al.,* 1996) and were cloned into plasmid pCR2.1 using the TA-cloning kit (Invitrogen Inc., Carlsbad, CA) producing plasmid pCR/HveAs. The cloned HveAs gene was sequenced using dideoxy-chain termination methodology, and the predicted primary structure of the protein was obtained (GenBank Accession No. AF147720). The HveAs gene was isolated from plasmid pCR/HveAs after restriction with *Eco*RI and cloned into plasmid pcDNA3.1Zeo^{+} in the coding and noncoding orientations producing plasmids pCMV/HveAs and pCMV/sAevH, respectively. The extracellular domain of HveAs (first 202 amino acids) was fused to a DNA fragment specifying the Fc portion of the mouse IgG

heavy chain using PCR-assisted splice-overlap-extension (Chouljenko *et al.,* 1996) and cloned into plasmid pCR3.1 using the eukaryotic TA-cloning kit (Invitrogen, Inc.), producing plasmid pCR/HveAs:Fc. Also, this portion of the HveAs gene was cloned into plasmid pMALc2 (NEB, Beverly, MA), producing plasmid pMAL/HveAs. Plasmid pTF9202 was constructed by inserting the gK gene, after amplification with primer pair UL52KpnI2/gK*Spe*I (5′-AACACCAACACTAGTGGTGGATGTCCTTATACC-3′) into the unique *Kpn*I and *Spe*I sites of pTF9201 (Foster *et al.,* 1999). This plasmid was used to transfer the EGFP gene into the d27–1 viral genome.

Construction of the HSV-1 (KOS)/EGFP recombinant virus

Plasmid pTF9202 specifying the CMV-EGFP gene cassette flanked by homologous HSV-1 sequences was transfected into 50% confluent Vero cells using Lipofectamine (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Twenty-four hours post transfection (h p.t.), the cells were infected at an m.o.i. of 10 with d27–1 virus (ICP27-null) as described previously for the generation of the gK null virus, ΔgK (Jayachandra *et al.,* 1997). At 48 h p.i., the cells were freeze-thawed three times, and the resultant virus stocks were plated onto Vero cells and overlaid with agarose. Virus plaques were visualized by fluorescent microscopy, individual virus plaques were isolated, and virus was plaque purified five times on Vero cells.

Diagnostic PCR

Primer pairs UL52KpnI2/gKTr2, and UL52KpnI2/CMVBamHI were used to confirm the EGFP gene insertion as described previously (Foster *et al.,* 1999). Primer pair UL52KpnI2/d27–1α was used to detect the presence of contaminating d27–1 viral DNA. Reaction conditions for UL52KpnI2/gKTr2 and UL52KpnI2/d27–1α pairs were 98°C for 3 s and 72°C for 7 min, repeated for 30 cycles using rTth XL-Polymerase (Perkin–Elmer, Inc.). Reaction conditions for the UL52KpnI2/CMVBamHI primer pair were 98°C for 3 s and 72°C for 3 min, repeated for 30 cycles using rTth XL-Polymerase.

Construction of Chinese hamster ovary (CHO) cell lines transformed with the HveAs or HveAs:Fc genes

Plasmid pCMV/HveAs, pCMV/sAevH, and pCR/HveAs:Fc were transfected into CHO cells using LipofectAmine and transformed cells were selected using Zeocin for the first two plasmids (Invitrogen, Carlsbad, CA) and G418 (Sigma, Inc.) for the pCR/HveAs:Fc plasmid. Individual CHO colonies were isolated, expanded, and tested for the presence of the HveAs gene using PCR and Southern blotting.

Production of anti-HveAs-specific antibodies

Plasmid pCMV/HveAs was used to generate mouse anti-HveAs antibodies by direct injection into mice (genetic immunization). Purified plasmid DNA was inoculated intramuscularly into mice five times approximately every 4 weeks. HveAs was expressed in *E. coli* as a fusion protein with the maltose binding protein (MBP) by plasmid pMAL/HveAs and purified according to the manufacturer's instructions (NEB, Beverly, MA). Anti-HveAs antibodies were tested in immunoblots against the MBP/HveAs fusion protein essentially as described previously (Foster *et al.,* 1999).

Production of HveA:Fc by CHO-transformed cells

HveAs:Fc protein secreted in supernatant fluids of $\sim 10^7$ CHO/HveAs:Fc-transformed cells was purified using protein A sepharose beads. Samples were resuspended in SDS–PAGE sample buffer and electrophoretically separated in a 10% SDS–polyacrylamide gels. Following electrophoretic separation, proteins were electrotransferred to nitrocellulose membranes. Blots were blocked against nonspecific binding and incubated with horse radish peroxidase conjugated goat anti-mouse secondary antibody (Pierce, Rockford, IL) at a 1:50,000 dilution in TBS-T. Blots were visualized by autoradiography using the Pierce SuperSignal chemiluminescent detection kit (Pierce) as per the manufacturer's instructions.

Phase-contrast and fluorescent microscopy

Subconfluent cell monolayers in 24-well plates were infected with the KOS/EGFP virus at an m.o.i. of 0.02 for visualization of individual viral plaques by either phase contrast or fluorescence microscopy essentially as described previously for the ΔgK/EGFP virus (Foster *et al.,* 1999). Viral entry experiments were performed at m.o.i. of 10 and visualized by fluorescence microscopy at 24 h p.i.

FACS analysis

CHO-transformed cells expressing HveAs or Vero cells were fixed with paraformaldehyde and incubated with anti-HveAs antibody for 1 h at 4°C. Next cells were washed three times with PBS buffer, incubated with goat-anti-mouse FITC-conjugated secondary antibody (Sigma, Inc.), and analyzed by FACS. KOS- and KOS/EGFP-infected Vero cells were analyzed by FACS at 24 h p.i. as described previously (Foster *et al.,* 1999).

Inhibition of virus entry by anti-HveAs antibody and soluble HveA-Fc protein

CHO/HveAs cells were incubated with either pre-immune or anti-HveAs serum for 1 h at room temperature immediately prior to infection with KOS/EGFP at a m.o.i. of 10. One milliliter of supernatant fluids from either CHO/HveAs:Fc or CHO cells transformed with pCR3.1

(control) was mixed with 1 ml of KOS/EGFP virus stock (1×10^8 PFU/ml) and incubated at 4°C for 1 h immediately prior to infection. The amount of virus used in these experiments was $\sim 10^6$ PFU per 10^5 CHO/HveAs cells.

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