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The soluble amino-terminal region of HVEM mediates efficient herpes simplex virus type 1 infection of gD receptor-negative cells

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Abstract

Background: Previous studies from our own and other labs reported the surprising finding that the soluble V domain of the herpes simplex virus type 1 (HSV-1) entry receptor nectin-1 can both block HSV infection of receptor-bearing cells and mediate infection of receptor-deficient cells. Here we show that this property is not unique to nectin-1. We generated a pair of truncated, soluble forms of the other major HSV-1 entry receptor, herpes virus entry mediator (HVEM or HveA), and examined its effects on HSV-1 infection of receptor-deficient cells.

Results: In cultures of CHO-K1 cells, sHveA₁₀₂ comprising the two amino-terminal cysteine-rich pseudorepeats (CRPs) of HVEM enabled infection of greater than 80% of the cells at an MOI of 3, while sHveA₁₆₂ comprising the complete ectodomain failed to mediate infection. Both sHveA₁₀₂ and sHveA₁₆₂ blocked infection of CHO-K1 cells stably expressing HVEM in a dose-dependent manner, indicating that both were capable of binding to viral gD. We found that sHveA₁₀₂-mediated infection involves pH-independent endocytosis whereas HSV infection of HVEM-expressing CHO-K1 cells is known to be pH-dependent.

Conclusions: Our results suggest that the C-terminal portion of the soluble HVEM ectodomain inhibits gD activation and that this effect is neutralized in the full-length form of HVEM in normal infection.

Keywords: HSV-1, HVEM/HveA, gD, Soluble entry receptor

Background

Herpes simplex virus type 1 (HSV-1) infects a broad range of mammalian cells, including epithelial cells, lymphocytes, and post-mitotic neurons. Initial HSV attachment to host cells is mediated by the binding of viral envelope glycoproteins C (gC) and gB to ubiquitous heparan sulfate moieties on the surface of cells [1-3]. While not essential [4], these interactions facilitate the binding of glycoprotein D (gD) to one or more of its cognate cell-surface receptors, nectin-1 (HveC), HVEM (HveA), and 3-O-sulfated heparan sulfate (3-OS HS) [5-7]. Binding to these entry receptors causes conformational changes in the gD ectodomain that signal activation of the downstream effectors of HSV entry, gB and gH/gL, the proximal mediators of membrane fusion and

capsid delivery into the cytoplasm [8-12]. Recent studies have also demonstrated that PILR α (paired immunoglobulin-like type-2 receptor) and non-muscle myosin heavy chain IIA can function as HSV-1 entry co-receptors through interaction with gB [13,14].

The absolute dependence of HSV-1 infection on gD binding to a cognate receptor indicates that the tropism of the virus is determined at least in part by the distribution of gD receptors. Nectin-1 is a member of the immunoglobulin superfamily and is expressed on many cell types, including fibroblasts, epithelial cells and neurons [15]. Nectins function as intercellular adhesion molecules localized to cadherin-based adherens junctions [16]. The variable (V) domain of nectin-1 is sufficient for binding to gD and the initiation of fusion between the virus envelope and cell membranes [17]. HVEM is a member of the tumor necrosis factor receptor (TNFR) superfamily and is expressed in hematopoietic cells and lymphoid tissues such as spleen and thymus [18,19]. HVEM contains

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four cysteine-rich pseudorepeats (CRPs) characteristic of members of the TNFR family in its ectodomain and functions as a mediator of HSV-1 and HSV-2 entry mainly into human lymphocytes [6,19]. The natural ligands for HVEM include LIGHT, lymphotoxin alpha (Lt- α), B- and T-lymphocyte attenuator (BTLA), and CD160 [20]. The contribution of the third gD receptor, 3-OS HS, to the broad HSV-1 tropism is not as clearly defined since this glycosaminoglycan modification is not easily detectable by immunological or other methods. However, novel approaches are beginning to reveal the role of this receptor [21].

X-ray crystallography has shown that HVEM binds to a flexible hairpin at the amino terminus of gD [8]. A variety of mutations in this region, including Q27P originally identified in KOS-rid1 virus [22], abolish HVEM binding [23,24]. Using a series of truncated forms of HVEM, Whitbeck and colleagues demonstrated that the two N-terminal CRPs of HVEM are required and sufficient for binding to HSV-1 gD [25]. In their study, HveA(120 t), consisting of the first and second CRP, showed full gD binding activity by competition ELISA and blocked HSV entry into CHO cells expressing HVEM.

We previously reported that the V-domain of nectin-1 as a soluble form can mediate efficient virus entry into HSV-resistant CHO-K1 cells that lack gD receptors [26]. Here, we have investigated whether soluble forms of the HVEM ectodomain have a similar ability. To this end, we constructed soluble recombinant proteins consisting of the first two CRPs (sHA₁₀₂) or the full ectodomain (sHA₁₆₂) of HVEM. While both soluble proteins inhibited HSV infection of HVEM-expressing CHO-K1 cells, only sHA₁₀₂ mediated virus entry into receptor-negative CHO-K1 cells. Infection mediated by sHA₁₀₂ was highly efficient, involved specific binding of sHA₁₀₂ to viral gD, required heparin-sensitive virus attachment to the cells, and took place by a pH-independent endocytic mechanism as opposed to the pH-dependent mechanism mediating HSV infection of HVEM-expressing CHO cells [27]. Our results indicate that the C-terminal portion of the HVEM ectodomain contains sequences that inhibit gD activation and suggest a context effect allowing gD activation by full-length HVEM, but not the soluble ectodomain, in the acidic milieu of endosomes.

Results

Production of soluble gD receptors

We constructed expression plasmids for two His₆-tagged soluble HVEM proteins, sHA₁₀₂ containing the gD-binding first two CRPs (102 aa), and sHA₁₆₂ comprising the full ectodomain (162 aa) (Figure 1A). Soluble proteins were produced by transfection of 293 T cells and purified from the culture supernatant, as previously described [26]. The molecular size sHA₁₀₂ was approximately 15 kDa

(Figure 1B, C), somewhat larger than the predicted size of 11 kDa most likely due to N-linked glycosylation in CRP2 [28]. sHA₁₆₂ migrated predominantly as a 38 kDa species, significantly larger than the predicted 18 kDa for the monomeric form, suggesting incomplete denaturation of the dimeric form that predominates in solution [28].

Soluble HVEM-mediated HSV-1 infection of CHO-K1 cells

We determined whether sHA₁₀₂ and sHA₁₆₂ could mediate HSV-1 entry into CHO-K1 cells that are resistant to infection due to the absence of entry receptors. KOS/tk12, expressing LacZ from the viral thymidine kinase (tk) locus in a wild-type (strain KOS) viral background [29], was preincubated at a multiplicity of 3 pfu/cell with CHO-K1 cells in suspension for 1 h at 4°C. Increasing concentrations (0-1,000 nM) of sHA proteins were then added and the cells were incubated for 1 h at 37°C, plated, and incubated for another 7 h at 37°C prior to measurement of β -galactosidase activity by quantitative ONPG assay. As shown in Figure 2A, dose-dependent infection was observed in the presence of sHA₁₀₂, whereas sHA₁₆₂ failed to mediate infection. At an MOI of 9, infection reached a plateau at 500 nM sHA₁₀₂ (Figure 2B), suggesting that either the amount of virus or the number of cells became limiting at this concentration. A similar level of infection was observed at an MOI of 3 with 1,000 nM sHA₁₀₂, arguing for the second interpretation. Indeed, titration of X-gal stained cells showed infection of 90-100% of the cells at an MOI of 9 at 500-1,000 nM sHA₁₀₂ (Figure 2C). These results demonstrated that the known gD binding region of HVEM consisting of CRPs 1 and 2 was sufficient to mediate HSV entry into receptor-deficient cells. As shown in Figure 2D, the efficiency of sHA₁₀₂-mediated entry was comparable to that mediated by the soluble V domain of nectin-1 (sNec₁₂₃) described previously [26].

Soluble HVEM blocks HSV infection of HVEM-expressing CHO cells

Soluble forms of HVEM and nectin-1 have been shown to inhibit HSV-1 entry through the cognate cellular receptors by competition for gD binding [28]. To determine whether the distinct entry-mediating activities of our sHA₁₀₂ and sHA₁₆₂ preparations correlated with their ability to inhibit virus entry via HVEM, we carried out competitive inhibition assays on CHO cells expressing HVEM. Cells were infected with KOS/tk12 in the presence of soluble gD receptors and β -galactosidase expression was measured by ONPG assay. As shown in Figure 4, expression decreased with increasing amounts of sHA₁₀₂ and sHA₁₆₂, indicating dose-dependent entry inhibition. While 1,000 nM of sHA₁₀₂ was required to inhibit KOS/tk12 infection by approximately 50%, sHA₁₆₂ reached this level of inhibition at 250 nM. The greater blocking efficiency of sHA₁₆₂ is in sharp contrast to its lack of entry-mediating activity on

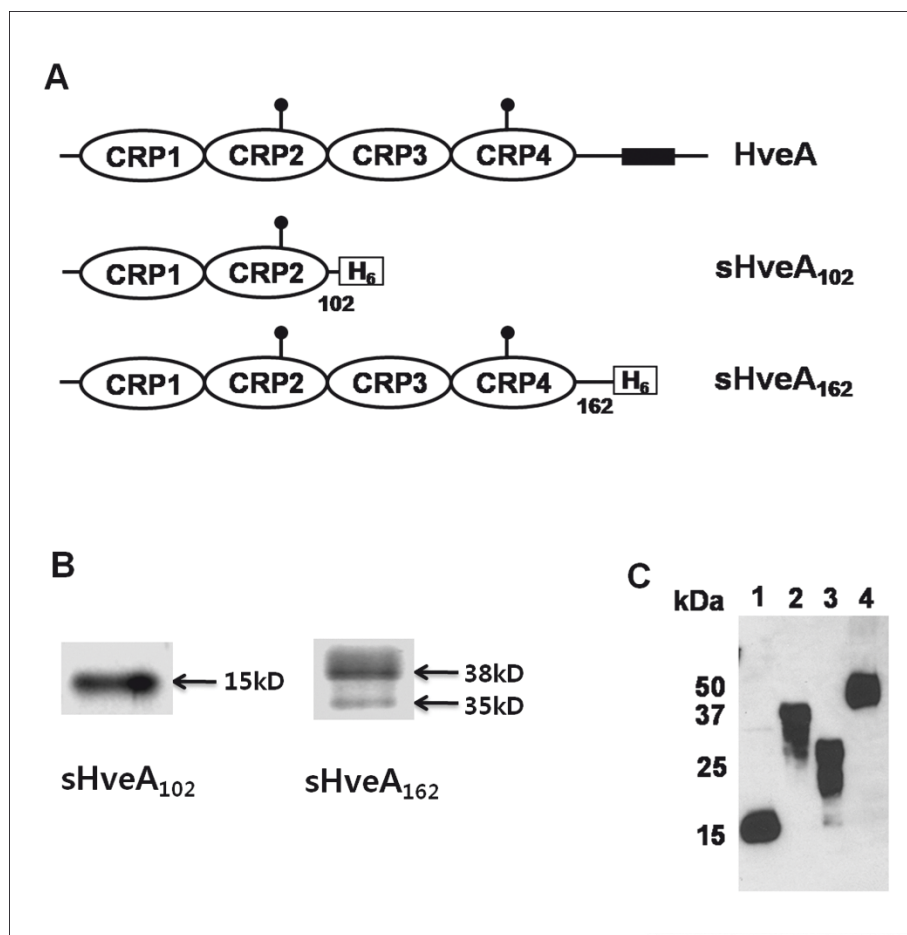


Figure 1 Structure and expression of soluble HVEM proteins. (A) Schematic representations of full-length HVEM (HveA) and the truncated HVEM proteins produced for this study. Lollipop symbols indicate glycosylation sites and numbers indicate amino acid positions relative to position 1 of mature HVEM. CRP, cysteine-rich pseudorepeat; H₆, six-histidine tag. (B) Silver-stained SDS-PAGE gels of soluble protein samples. (C) Western blot detection of purified recombinant soluble proteins using anti-His tag antibody. Lane 1, sHA₁₀₂; lane 2, sHA₁₆₂; lane 3, sNec1₁₂₃ used in Figure 2D; lane 4, sgD₂₈₇ used in Figure 3.

receptor-deficient cells. One possible explanation is that CRP 3 and 4, when not anchored in the cell membrane, sterically limit the activation of gD involving partial unfolding to expose its effector/pro-fusion domain [10,12].

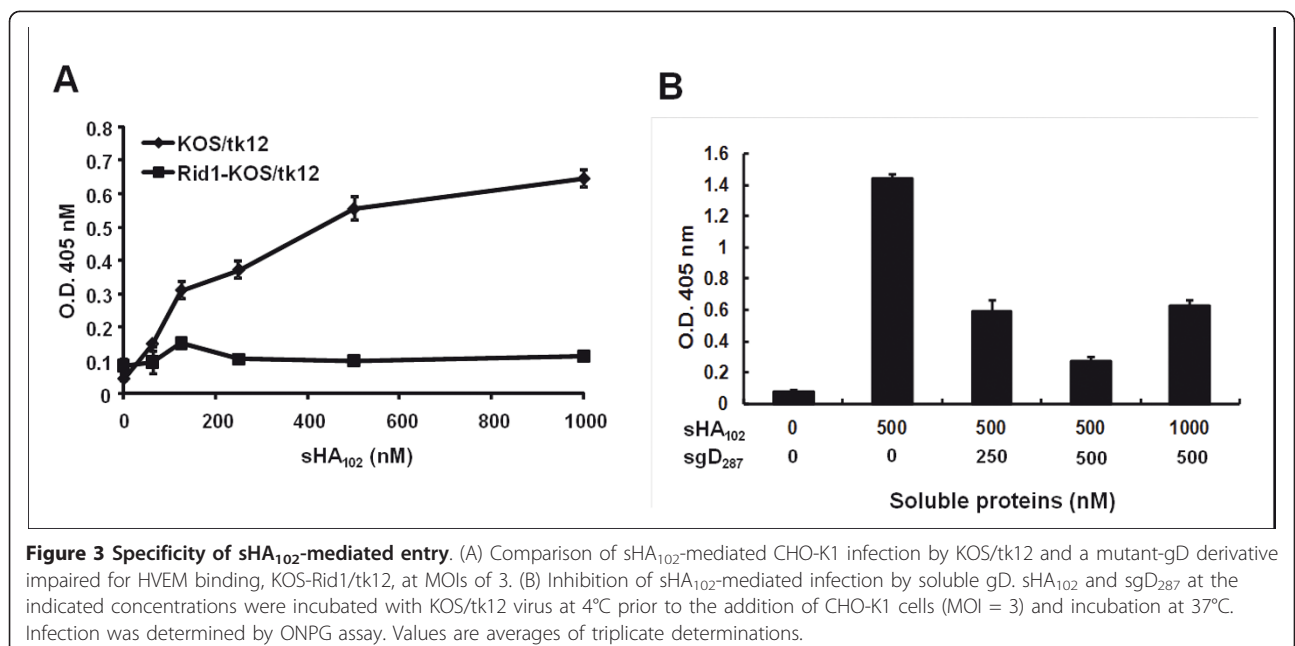
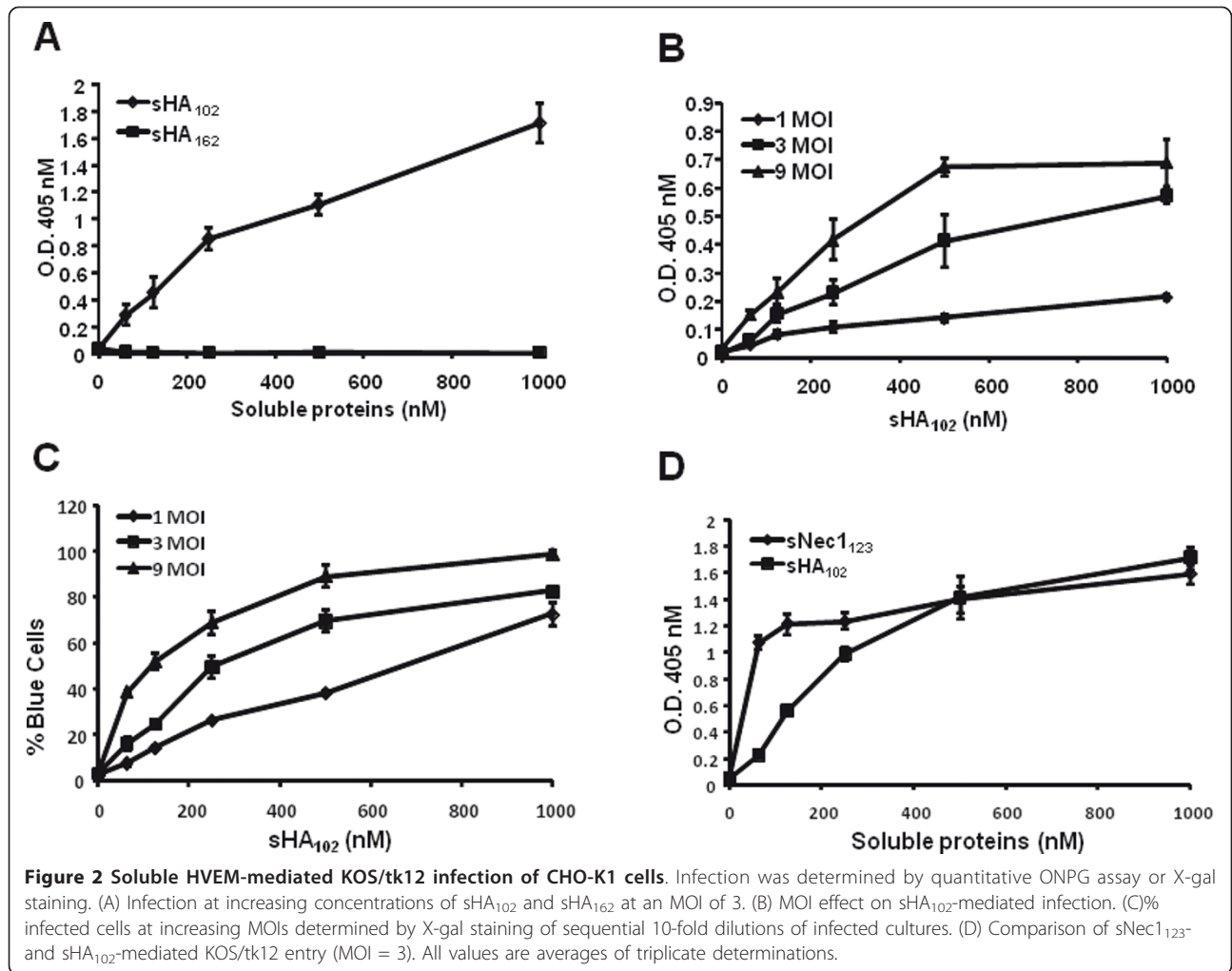
Specific interaction of sHA₁₀₂ with viral gD

We determined whether sHA₁₀₂-mediated virus entry into CHO-K1 cells requires specific interaction with gD. As illustrated in Figure 3, while sHA₁₀₂ promoted entry of KOS/tk12 into CHO-K1 cells, little entry was observed by an isogenic mutant virus, KOS-Rid1/tk, that has an amino-acid substitution in gD preventing interaction with HVEM [29]. In addition, we observed that preincubation of sHA₁₀₂ with soluble gD ectodomain (gD₂₈₇; Figure 1C) inhibited virus entry into CHO-K1 cells in a sgD₂₈₇ dose-dependent manner. When the concentration of sHA₁₀₂ was augmented up to 100 nM, the viral infection was

recovered to 2 fold (Figure 3). Together, these results indicated that sHA₁₀₂-mediated HSV entry into CHO-K1 cells occurred through specific binding to viral gD.

Requirement of heparan sulfate binding for sHA₁₀₂-mediated infection

HSV attachment is mediated by the binding of gB and gC to cell-surface glycosaminoglycans, particularly heparan sulfate [30]. To determine whether heparan sulfate binding was required for sHA₁₀₂-mediated HSV entry, infections were performed in the presence of 25 μg/ml heparin. As shown in Figure 5, infection was reduced at all MOIs tested, including more than 200-fold at an MOI of 10. These results indicated that virus attachment to cell-surface heparan sulfate is necessary for efficient sHA₁₀₂-mediated HSV entry, as previously observed for soluble nectin-1-mediated infection [26].



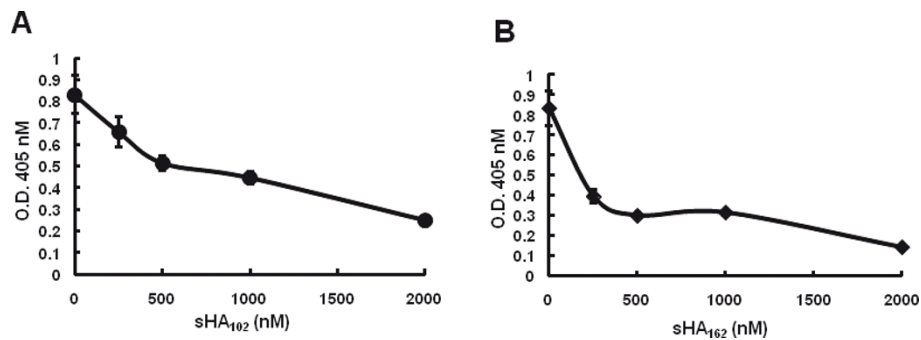


Figure 4 Inhibition of KOS/tk12 infection of HVEM-expressing CHO cells by soluble HVEM proteins. Increasing amounts of sHA₁₀₂ (A) or sHA₁₆₂ (B) were preincubated with the virus for 1 h at 4°C prior to infection of CHO-HVEM-12 cells (MOI = 1) for 1 h at 37°C. The cells were washed and infection was measured 7 h later by ONPG assay. Values are averages of triplicate determinations.

Pathway of sHA₁₀₂-mediated HSV entry into CHO-K1 cells

Previous reports have shown that HSV enters into CHO cells expressing human nectin-1 or HVEM via a low pH-dependent endocytic pathway [27]. To examine whether the same entry pathway is used in sHA₁₀₂-induced HSV-1 infection of CHO-K1 cells, we treated the cells with hypertonic medium or lysosomotropic agents. Medium containing 0.3 M sucrose reduced sHA₁₀₂-mediated KOS/tk12 infection approximately 5-fold (Figure 6B), similar to results reported for HSV infection of HVEM-expressing CHO cells (Figure 6A) [27], indicating an important role for active endocytosis in entry mediated by sHA₁₀₂. To determine whether endosomal acidification was required for infection, CHO-K1 cells were pre-treated with increasing concentrations of NH₄Cl or monensin, and incubated with KOS/tk12 and sHA₁₀₂ in the continued presence of these agents. The results showed no inhibition of

sHA₁₀₂-mediated infection while infection of CHO-HVEM cells was sensitive to these agents (Figure 6C, D). Thus, unlike HSV infection of HVEM-expressing CHO cells, sHA₁₀₂-mediated CHO-K1 infection appears to take place by a low pH-independent endocytic mechanism.

Discussion

In this study, we demonstrate that a soluble gD receptor consisting of the N-terminal 102 amino acids of HVEM is an efficient mediator of HSV-1 infection of receptor-negative, HSV-resistant CHO-K1 cells. In contrast, sHA₁₆₂, comprising the complete HVEM ectodomain, did not enable viral entry although both sHA₁₀₂ and sHA₁₆₂ blocked virus infection of receptor-bearing CHO cells. We suggest that sHA₁₆₂ binds to viral gD but prevents the folding of gD into an active conformation that can trigger the fusion process. Using inhibitors of receptor- and pH-dependent endocytosis, we obtained evidence that sHA₁₀₂-mediated infection of CHO-K1 cells occurs by pH-independent endocytosis, unlike the low pH-dependent endocytic mechanism responsible for infection of CHO-HVEM cells [27].

Previous studies have reported inhibition of HSV infection of CHO-HVEM cells by the soluble ectodomain of HVEM, alone [HVEM (200 t)] or as a fusion with the Fc region of rabbit immunoglobulin G (IgG) heavy chain [6,28]. In a later study, Whitbeck and colleagues also reported the ability of HVEM (200 t) to promote HSV entry into receptor-deficient CHO-K1 cells [31]. Although our sHA₁₆₂ had essentially the same primary sequence as HVEM (200 t), we did not observe an entry-promoting effect of sHA₁₆₂ on CHO-K1 cells. A potentially significant difference with our study is that Whitbeck et al. [31] used a low speed centrifugation technique referred to as spinoculation to augment HVEM(200 t)-mediated virus infection. Spinoculation has been used with HSV and other enveloped viruses to bring virion

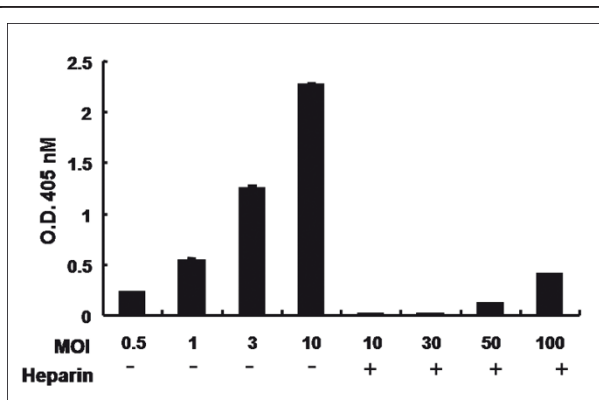


Figure 5 Heparin inhibition of sHA₁₀₂-mediated KOS/tk12 entry. Virus and heparin (25 µg/ml) were pre incubated for 30 min at 4°C, CHO-K1 cells were added, and the incubation was continued for another 30 min prior to the addition of sHA₁₀₂ (500 nM), infection as in previous figures, and ONPG assay. Values are averages of triplicate determinations.

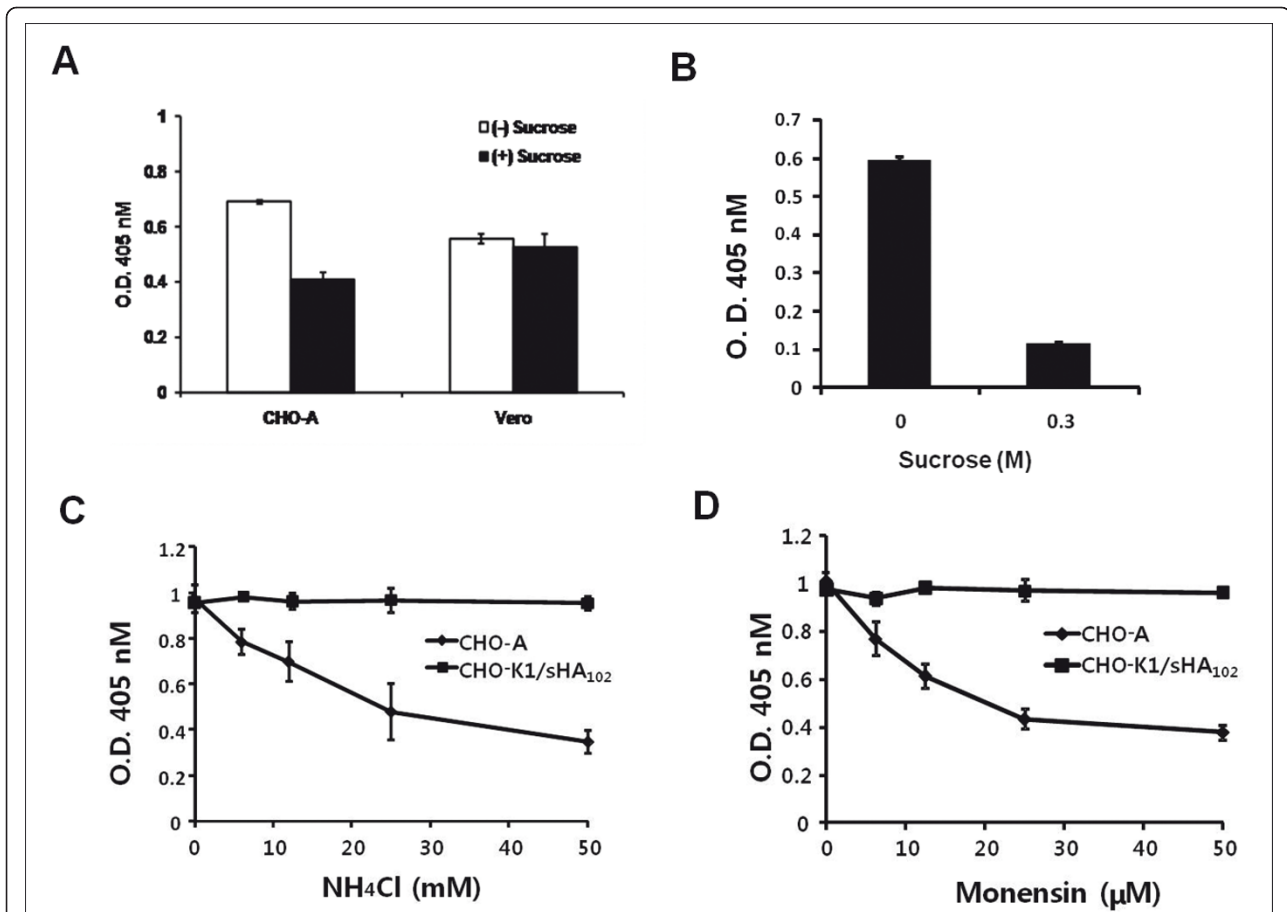


Figure 6 Effect of agents that inhibit endocytic virion uptake or low pH-dependent infection. (A) CHO-A and Vero cells were incubated with KOS/tk12 (MOI = 3) in hypertonic medium containing 0.3 M sucrose for 1 h at 4°C and infections were performed in the continuous presence of sucrose followed by ONPG assay. (B) CHO-K1 cells were incubated with KOS/tk12 (MOI = 3) in hypertonic medium containing 0.3 M sucrose for 1 h at 4°C, sHA₁₀₂ was added to 500 nM, and infections were performed in the continued presence of sucrose followed by ONPG assay. (C, D) CHO-K1 cells and CHO-HVEM-12 cells (CHO-A) were pretreated with the indicated amounts of NH₄Cl (C) or monensin (D) for 30 min at 4°C prior to KOS/tk12 attachment at 4°C (MOI = 3), addition of sHA₁₀₂ to 500 nM (CHO-K1 cells only), and infection as in previous figures in the continued presence of the respective drugs. Infection was measured by ONPG assay. Values are averages of triplicate determinations.

and cell membranes closely together [21,32-34], for example to bypass receptor-dependent virus attachment in studies demonstrating soluble receptor-mediated infection by avian sarcoma and leukosis viruses [35,36]. Since HSV attachment to cells is mediated mainly by viral envelope proteins other than gD, we performed our experiments without spinoculation while showing that heparin-sensitive virus attachment was essential under these conditions. We observed highly efficient sHA₁₀₂-mediated infection (> 80% infection at an MOI of 3), but entry mediated by the complete soluble ectodomain was essentially undetectable, suggesting that spinoculation in the study by Whitbeck et al. [31] had allowed the detection of HVEM (200 t) activity by concentrating the virus at the cell surface to raise the sensitivity of the assay. Nonetheless, the Whitbeck study reported a maximum HVEM (200 t)-mediated increase in infection of CHO-

K1 cells of 15.5-fold [5 µM HVEM (200 t); MOI = 2], substantially less than the increases we observed at lower sHA₁₀₂ concentrations (Figure 2). Thus, while sHA₁₆₂/HVEM (200 t) may not be inactive in infection of receptor-deficient cells, sHA₁₀₂ is clearly more effective.

The similar inhibition of CHO-HVEM infection by sHA₁₀₂ and sHA₁₆₂ indicated that the difference in entry-mediating activity between these two proteins is not due to differences in gD binding, suggesting that the extra sequences in sHA₁₆₂/HVEM(200 t) compared to sHA₁₀₂ negatively affect the efficiency of gD activation. The previously reported crystal structure of the gD ectodomain complexed with the HVEM ectodomain has shown that the N-terminal region of gD undergoes a conformational change during HVEM binding [8]. It is conceivable that sHA₁₀₂ either induces a more global change in the gD conformation or that the C-terminal portion of sHA₁₆₂/

HVEM(200 t) interferes with the efficient receptor-dependent unmasking of the gD effector/pro-fusion domain proposed by Krummenacher et al. [12] and Fusco et al. [10].

In the absence of gD receptors, HSV is internalized by CHO cells but fails to enter the cytoplasm and is degraded in lysosomes [37]. The presence of gD receptors allows pH-dependent viral capsid escape from endosomes and release into the cytosol [37]. Whitbeck et al. [31] have described evidence that HVEM (200 t) “primes” gD for activation of the membrane fusion machinery, but that a mildly acidic pH is required in CHO cells as a second signal for fusion initiation. Surprisingly, our results using NH₄Cl or monensin to neutralize the endosomal pH did not show a reduction in sHA₁₀₂-mediated infection although virus uptake by endocytosis was indicated by reduced infection in media containing 0.3 M sucrose. Thus it appears that the second signal is not required for sHA₁₀₂ activation of gD. At the opposite end, it appears that endosome acidification as the second signal is not sufficient for efficient activation of gD by sHA₁₆₂/HVEM (200 t). Thus our results suggest that both situations differ from normal virus entry via full-length HVEM. It is of interest that several examples have been reported of cell-surface HVEM failing to serve as a functional HSV entry receptor [38-40], raising the possibility that these cells lack a mechanism to counteract interference by CRPs 3 and 4 with gD activation by full-length HVEM. Our study suggests that sHA₁₀₂ may be an efficient tool to enable HSV vector-mediated delivery of therapeutic genes to receptor-deficient cells.

Conclusions

Here we have demonstrated a soluble gD receptor, containing N-terminal 102 amino acids of HVEM, for efficient HSV infection into HSV receptor-negative, HSV-resistant CHO-K1 cells. This study is consistent with two other soluble forms of gD receptors, sNec1₁₂₃ and soluble 3-OS HS. We can improve the usage of sHA₁₀₂ by fusing it with ligands or receptors of disease related antigens as a promising molecule for retargeting of natural tropism of HSV-1. This retargeting strategy can be combined to create specific recognition by genetic modifications of the virion envelope for incorporation of targeting moiety.

Methods

Cells and viruses

African green monkey kidney (Vero) cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modification of Eagle minimal medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Chinese hamster ovary cells (CHO-K1, ATCC) were maintained in Ham's F-12 K medium with 10% FBS, 100 U/ml penicillin, and 100 ug/ml

streptomycin (Invitrogen, Carlsbad, CA). CHO-HVEM-12 cells (Montgomery et al., 1996) are CHO-K1 derivatives constitutively expressing full-length human HVEM and were kindly provided by Patricia G. Spear (Northwestern University, IL, USA). Both cell lines were grown in Ham's F-12 K medium supplemented with 10% FBS and 400 µg/ml G418 (Invitrogen).

Wild-type and mutant gD β-galactosidase reporter viruses KOS/tk12 and KOS-Rid1/tk12 [29] were kindly provided by Patricia G. Spear and were propagated and titered on Vero cells.

Soluble protein expression constructs

Sequences encoding sHA₁₀₂ and sHA₁₆₂ were amplified by PCR on HVEM plasmid pBEC14 ([6]; a kind gift from Patricia Spear) using the following primers: sHA₁₀₂/sHA₁₆₂ sense primer, 5'-CCC AAG CTT GCC ACC ATG GAG CCT CCT GGA GAC TGG GGG CC-3'; sHA₁₀₂ anti-sense primer, 5'-CCC CTC GAG CTA ATG GTG ATG GTG ATG GTG AGC GCG GCA CGC GGC GCA-3'; sHA₁₆₂ anti-sense primer, 5'-CCC CTC GAG CTA GTG GTG GTG GTG GTG GTG GGA GCT GCT GGT CCC AGC-3'. Primers were designed to provide a Kozak translational initiation sequence at the start of the open reading frame (ORF) and six histidine residues followed by a TAG stop codon at the end. Underlined sequences indicate recognition sites for the restriction enzymes *Hind* III and *Xho* I. Following digestion with *Hind* III plus *Xho* I, each PCR product was cloned into pcDNA3.1 (+) (Invitrogen), yielding mammalian expression constructs pHA₁₀₂ and pHA₁₆₂.

Expression plasmids for sNec1₁₂₃ and sgD₂₈₇ were as previously described [26].

Production and purification of His fusion proteins

Soluble proteins were expressed in 293 T cells and purified using the Probond™ resin purification system (Invitrogen) according to the manufacturer's protocol. Briefly, DNA transfections were performed with LipofectAmine Plus reagent (Invitrogen) and the cells were then cultured in serum-free media. Culture supernatants were harvested at 72 h post-transfection, mixed with Probond resin, and incubated for 1 hr at 4°C. The resin was washed and bound proteins were eluted.

SDS-PAGE and Western blotting

Purified soluble proteins were electrophoresed on a 12% polyacrylamide-sodium dodecyl sulfate (SDS) gel and blotted onto Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked with a solution of 3% skim milk in PBS/0.1% Tween-20, and incubated for 1 h at room temperature with anti-His antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

diluted in blocking buffer. The membrane was washed three times and treated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) for 1 h at room temperature. Peroxidase activity was detected using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ).

HSV entry assay

CHO-K1 cells in suspension (6×10^4 cells/well) were preincubated with KOS/tk12 virus in PBS in a total volume of 40 μ l for 1 h at 4°C on a rocking device. Increasing concentrations of soluble proteins (0 - 1,000 nM) were added to the virus/cell mixture and incubated for 1 h at 37°C. The cells were collected by centrifugation, washed twice with PBS, resuspended in 40 μ l of Ham's F-12 K medium, and plated in a single well of a 96 well plate. After continued incubation at 37°C for 7 h, virus entry was detected by staining with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) or by colorimetric assay (ONPG, *o*-nitrophenyl- β -D-galactopyranoside), as previously described [41]. Briefly, infected cell monolayers were fixed with 0.2% glutaldehyde (Sigma) for 15 min at room temperature (RT), washed with PBS, and incubated with 0.2 mg/ml X-gal (Sigma); % infection was determined by staining of sequential 10-fold dilutions of infected cells. For the colorimetric assay, infected monolayers were washed with PBS and lysed in 150 μ l of 1% NP-40, 1 mM MgCl₂, 50 mM β -mercaptoethanol, and 4 mg/ml ONPG (Sigma). Lysates were incubated at 37°C until a yellow color developed, and absorbance was measured at 405 nM.

Infection inhibition by soluble receptors

Different concentrations of soluble receptors (0-2,000 nM) were preincubated with 6×10^4 pfu of KOS/tk12 in a total volume of 40 μ l for 1 h at 4°C. CHO-HVEM-12 cells (6×10^4 cells/well) were added and the mixture was incubated for 1 h at 37°C. The cells were then collected, washed twice with PBS, resuspended in 40 μ l of Ham's F-12 K medium, and incubated for 7 h at 37°C. Infection was measured by colorimetric ONPG assay, as described above.

Infection inhibition by sgD₂₈₇

Experiments to determine the sensitivity of sHA₁₀₂-mediated infection to competition for sHA₁₀₂ binding with soluble gD were performed essentially as described previously for sNec₁₂₃ (Kwon et al., 2006). Briefly, various amounts of sHA₁₀₂ and sgD₂₈₇ were incubated with KOS/tk12 in PBS for 1 h at 4°C on rocking device. A total of 5×10^5 CHO-K1 cells (MOI = 3) were added and the mixture was incubated for 1 h at 37°C. The cells were collected, washed, plated with complete medium, and incubated for another 7 h. Infection was measured by ONPG assay.

Heparin inhibition

Increasing amounts of KOS/tk12 were preincubated with 25 μ l/ml heparin for 30 min at 4°C, CHO-K1 cells were added, and the incubation was continued for another 30 min. sHA₁₀₂ to a final concentration of 500 nM was then added and the mixture was incubated for 1 h at 37°C. The cells were washed, plated, incubated with complete medium for 7 h at 37°C, and infection was determined by ONPG assay.

Endocytosis inhibition assays

KOS/tk12 (MOI = 3) was preincubated with CHO-K1 cells for 30 min at 4°C in the presence of 0.3 M sucrose, sHA₁₀₂ (500 nM final concentration) was added, and the mixture was incubated for 1 h at 37°C. Alternatively, CHO-K1 cells were incubated with different concentrations of ammonium chloride (NH₄Cl) or monensin (Sigma) for 30 min at 4°C, virus was added and the incubation continued for another 30 min at 4°C, and sHA₁₀₂ was added with incubation for 1 h at 37°C. In each situation, the cells were washed, plated, and incubated for 7 h at 37°C in complete medium containing the respective inhibitory agents. Virus entry was measured by ONPG assay.

Statistical analysis

All results were performed in triplicate and the values are expressed as means \pm SD. P values were calculated by using Student's *t* test, and statistical significance was defined as $P \leq 0.05$.

Abbreviations

BTLA: B- and T-lymphocyte attenuator; CRPs: Cysteine-rich pseudo repeats; FBS: Fetal bovine serum; gC: Glycoproteins C; gD: Glycoprotein D; HVEM or HveA: Herpes virus entry mediator; HSV-1: Herpes simplex virus type 1; Lt- α : Lymphotoxin alpha; ONPG: *o*-nitrophenyl- β -D-galactopyranoside; ORF: Open reading frame; PILRa: Paired immunoglobulin-like type-2 receptor; Tk: Thymidine kinase; TNFR: Tumor necrosis factor receptor; X-gal: 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 3-OS HS: 3-O-sulfated heparan sulfate.

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Authors' contributions

HB and HK designed the research; HB and YN performed research; JK analyzed data; HB and HK contributed to drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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