

**The Production and Characterization of a Mutant Form of Herpes
Virus Entry Mediator (HVEM)**

By

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This thesis for honors recognition has been approved for the Department of Natural Sciences.

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Abstract

Herpes Simplex Virus (HSV) entry is a multistep process involving viral glycoproteins and cellular receptors. Viral glycoproteins bind cell-surface glycosaminoglycans, but this binding is not sufficient for virus entry. A cellular co-receptor is also essential for virus entry. Herpes Virus Entry Mediator (HVEM), an integral membrane protein, is a co-receptor that can mediate HSV entry. Hybrid proteins composed of HVEM and CD40 have shown that only one specific region, Cysteine Rich Repeat Domain 1 (CRRD1), of HVEM is essential to mediate HSV entry. The purpose of this project was to identify amino acids within CRRD1 essential for HVEM-mediated HSV entry. Site-directed mutagenesis using long PCR was utilized to introduce mutation(s) into a mammalian expression vector encoding HVEM. Mutation(s) were constructed to change specific amino acids in CRRD1. Mutant vectors were transfected into mammalian cells that are normally resistant to HSV entry. Transfected cells were then challenged with reporter virus to assay for entry-mediating ability. Results show one mutant was created that has virus entry-mediating ability at a lower level than wild type HVEM. However, the mutation(s) carried by this mutant protein are not consistent with the mutations that were specifically designed to be inserted into the new HVEM protein. This thesis describes the creation and characterization of the new HVEM mutant.

Introduction

According to Parker (2000), a virus can be described as “a genetic element containing either RNA or DNA that replicates in cells but is characterized by having an extracellular state.” Viruses can vary in size from 0.02 to 0.3 μ m and the viral genome is usually smaller than 200kb. Viruses require a host to replicate in and have no free-living form making them obligate intracellular parasites. All viruses contain some form of nucleic acid that is incased in a protein coat or capsid. The capsid is usually made of a number of proteins arranged in repeating subunits, and these subunits come together to form larger complexes called capsomers. The combination of the viral nucleic acids and the capsid is called the nucleocapsid. In addition to being covered by a nucleocapsid, some viruses, such as herpes viruses, are also covered in a membranous structure called an envelope, which they use to help them enter host cells.

Common members of the herpes virus family include the Epstein-Barr virus, herpes simplex virus I and II, and the varicella-zoster virus. These viruses cause Burkitt’s lymphoma, chickenpox/shingles, and fever blisters, respectively. Some herpes viruses such as herpes simplex can remain dormant in nerve cells until conditions become favorable for reinfection while others such as the Epstein-Barr virus can cause cancerous tumors (Parker 2000).

Herpes Simplex I is about 150nm in diameter with a core of double stranded DNA. The Herpes Simplex I genome is made up of one 152,260bp piece of circular DNA that codes for at least 84 different proteins. Herpes Simplex I has an isohedral capsid made up of 162 capsomeres and is surrounded by both a fibrous tegument and an envelope covered with tiny spikes (Parker 2000).

All viruses need a way to enter living cells. Adenoviruses use fibers to bind to cells, and these fibers also interact with cell-surface integrins to facilitate entry (Wickham *et al.*, 1993). Human immunodeficiency virus can attach to cells because of binding of viral gp120 protein and cell CD4 protein (Dalglish *et al.*, 1984; Klatzmann *et al.*, 1984; Maddon *et al.*, 1988, all cited in Montgomery *et al.*, 1996). However, HIV entry requires the use of cofactors (Feng *et al.*, 1996; Deng *et al.*, 1996; Dragic *et al.*, 1996; Choe *et al.*, 1996; Doranz *et al.*, 1996; Alkhatib *et al.*, 1996, all cited in Montgomery *et al.*, 1996). Similarly, Herpes Simplex Virus (HSV) possesses an envelope that fuses with a host cell through the binding of viral and cellular receptors and eventually allows the penetration of viral DNA into the host cell (Wittels and Spear, 1991). Understanding this fusion process may lead to prevention of or treatment for HSV infection.

The entry into cells by HSV DNA is a multi-step process involving viral glycoproteins and cellular receptors. Viral glycoproteins B and/or C bind cell-surface glycosaminoglycans, but this binding is not sufficient for viral entry (Shieh *et al.*, 1992; Montgomery *et al.*, 1996). Viral glycoproteins B, D, H and L are required for entry of the viral genome into the host cell (reviewed in Spear *et al.*, 2000).

Specifically, Herpes Virus Entry Mediator (HVEM), a member of the tumor necrosis factor receptor (TNFR), and a co-receptor which mediates HSV entry, binds with viral glycoprotein D (Montgomery *et al.*, 1996). HVEM has an open reading frame consisting of 283 amino acids, a structure with four cysteine rich repeat domains, and is a membrane spanning protein with an N-terminal signal peptide and up to two potential sites for the addition of N-linked glycans (Montgomery *et al.*, 1996). CD40 protein, another member of the TNFR superfamily, also has four cysteine rich repeat domains and

identity (exact amino acid sequence match) of 25.4% and similarity (exact and amino acids of similar structure) of 37.1% with the amino acid sequence for HVEM (Montgomery *et al.*, 1996).

Data from studies using hybrid proteins composed of HVEM and CD40 have shown that only one specific region, Cysteine Rich Repeat Domain 1 (CRRD1) of HVEM is essential to mediate HSV entry (Wilson and Montgomery, 2002, unpublished data). The purpose of this thesis is to identify amino acids within CRRD1 essential for HVEM-mediated HSV entry. I hypothesized that changing specific amino acids within CRRD1 of HVEM would alter HVEM's ability to mediate virus entry. Site-directed mutagenesis was used to introduce single amino acid mutations in HVEM contained within a mammalian expression. The expression vector containing the mutated HVEM was amplified, transfected into, and expressed in cells that do not normally express HVEM. A virus infectivity assay was then conducted on the transfected cells to determine the ability of the mutated HVEM to mediate virus entry. Once specific amino acids necessary for HVEM mediated HSV entry have been identified, they may yield clues as to how the amino acid structure of CRRD1 of HVEM mediates HSV entry.

Materials and Methods

Primer Design

Forward and reverse primers were designed by Nathan T. Wilson using Primer Designer 4 for Windows 95 Version 4.00 Copyright (C) 1994-1999, and Clone Manager 5 for Windows 95 Version 5.10 Copyright (C) 1995-1999, both by Scientific & Educational Software. Primers were designed to introduce a specific mutation(s) into a mammalian expression vector encoding HVEM. The primers are designated pNW43F (forward) and pNW43R (reverse). Both primers were designed to change amino acid number 72 of HVEM from a glycine to an alanine and introduce a silent mutation and SacI restriction site at 471bp of HVEM. Primer pNW43F has a length of 26bp and a melting temperature of 87°C, while pNW43R has a length of 25bp and a melting temperature of 84°C. The primers are complimentary at 25bp.

The mammalian expression vector used was pBEC28. Dr. Rebecca I. Montgomery (University of Wisconsin-Madison) constructed pBEC28, a circular plasmid 6549bps in size. It carries the genes encoding Herpes Virus Entry Mediator, the immediate early human cytomegalovirus promoter, the G418/neomycin resistance gene, an ampicillin resistance gene, and a myc epitope/polyHis tag. Vector pBEC28 was constructed by inserting a NheI-EcoRI fragment from the HVEM cDNA into the same restriction sites of pcDNA3mycHISB, a mammalian expression vector from Invitrogen Corp.

Site-directed Mutagenesis

Site-directed mutagenesis was adapted from the Promega Quick Change Protocol. Polymerase Chain Reaction (PCR) conditions and PCR program times and temperatures

are described in Tables 1&2. The time and temperature conditions used were in general longer and of increased temperature in comparison to the original Quick Change Protocol to accommodate the high melting temperature of the primers. The Vent DNA polymerase enzyme from New England Biolabs was also used in place of Pfu DNA polymerase to increase the PCR efficiency. After PCR was conducted, amplified DNA (pDNA) was precipitated with ammonium acetate (AmOAc), then treated with enzyme DPN-I to remove methylated template DNA, and finally reprecipitated with AmOAc and extracted with phenol-chloroform and then ethanol. DNA was resuspended in Tris-EDTA buffer (TE) after the extractions.

Transformation

Competent *E. coli* (DH5 α) cells were transformed with the mutated plasmid DNA from the site-directed mutation PCR according to Maniatis *et al.* (1989). After transformation, cells were plated to LB plates containing 50 ug/mL Ampicillin. Colonies were grown overnight at 37°C after which they were picked and transferred to 4 ml of Terrific Broth (TB) containing 100ug/ml Ampicillin.

Small Scale DNA Preparation

Small scale DNA preparation/isolation was adapted from Maniatis *et al.* (1989) and conducted using the 4 ml colonies that showed growth overnight. About 1.4 ml of the bacterial culture was used in the DNA preparation. Plasmid DNA was separated from the bacterial cells using centrifugation, 100 μ g/ml lysozyme, and the detergent action of an SDS solution. Plasmid DNA was isolated from most other nucleic acids and the other components of the bacterial cell using phenol and phenol-chloroform extractions.

Ethanol was used to precipitate the plasmid DNA. Isolated plasmid DNA was resuspended in TE and RNAase enzyme was added to remove any remaining RNA.

Restriction Analysis of Small Scale DNA Preparation

Restriction digestion was done on the isolated plasmid DNA according to New England Biolabs protocol for digestion with the enzyme SacI. Total volume of reactions was 20 μ l with 5 μ l pDNA, 2 μ l 10X NEB buffer #1, 1 μ l of 100X BSA, 1 μ l of SacI enzyme, and 11 μ l of H₂O. In addition to the sample tested, the original mammalian expression vector, pBEC28, was used as a positive control. Samples were run on 1% agarose TBS gels and stained with ethidium bromide.

Large Scale DNA Preparation

Once bacteria containing a plasmid with the correct restriction pattern were isolated, large scale DNA preparation was conducted according to Maniatis *et al.* (1989), on these samples. Bacterial colonies containing plasmid were grown in a 250 or 500ml culture of TB with 500 μ l of 50mg/ml ampicillin. Cultures were incubated in a shaker at 37°C for approximately 24 hours. The plasmid DNA from the bacterial cells was harvested by lysis with lysozyme, SDS, and NaOH solutions. Centrifugation was used to separate cellular components at various stages of the lysis. Plasmid DNA was purified from other nucleic acids by polyethylene glycol and lithium chloride, RNAase, and phenol then phenol-chloroform extractions. Plasmid DNA was precipitated using AmOAc and ethanol. Precipitated plasmid DNA was resuspended and stored in TE. After large scale preparation, DNA samples were quantitated using UV spectrophotometry. Samples were diluted 1:100 and the absorbance of the diluted DNA solution was read at 320nm. The absorbance at 260nm was used to calculate the DNA

concentration. The ratio of the 260/280 absorbances was used to monitor the amount of RNA still present with the plasmid DNA.

Restriction Analysis of Large Scale DNA Preparation

Restriction digestion was performed according to New England Biolabs protocol for digestion with the enzyme SacI with modification of volumes and DNA concentrations from the restriction analysis of small scale DNA preparation. Total volume of reactions was 20 μ l with 1 μ l DNA of 10 μ g/ μ l DNA used, 2 μ l 10X NEB buffer #1, 1 μ l of 100X BSA, 1 μ l of SacI enzyme, and 16 μ l of H₂O. In addition to the sample tested, the original mammalian expression vector, pBEC28, was used as a positive control. Samples were run on 1% agarose TBS gels and stained with ethidium bromide.

Transfection

Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection, ATCC) were used as recipients for the mutated plasmid DNA. The transfection protocol was adapted from Life Technologies "Procedure for Transient or Stable Transfection of Adherent Cells." DNA for transfection was first diluted 1:10 in TE and then diluted accordingly to obtain 3 μ g of pDNA in 20 μ l for each transfection. Transfections were done when cells were 80-90% confluent on the bottom of their growth plates. Opti-Mem medium and LipoFECTAMINE (from Gibco) were used in the tranfection procedure. Transfections were incubated for about 16 hours at 37°C in CO₂ incubators. They were then supplemented with F12 medium containing 7% FBS (Fetal Bovine Serum) and antibiotics and incubated until 24 hours post infection. Cells were harvested after transfection with PBS-EDTA (Phosphate Buffered Saline) and Trypsin and collected by centrifugation. Cells were resuspended with the F12 medium described above and used

to inoculate 96 well plates and 60mm culture dishes. Cells were again incubated overnight at the conditions described above.

Virus Infection and β -galactosidase Assay.

Virus infection was conducted in triplicate. Virus was diluted in PBS-G-CS (PBS with 1% glucose and calf serum). Each infection had a volume of 50 μ l/well. The infection lasted six hours at 37°C and was conducted on a gently rocker in an incubator. The HSV-1(KOS)gL86 reporter virus was used to show viral entry. It is a β -galactosidase-expressing member of the HSV-1 KOS strain. It causes the production of β -galactosidase (β -gal) after it infects a cell and this production can be identified and used as an indication of virus entry by adding o-nitrophenyl β -D-glucopyranoside (ONPG). The concentrations of virus used were 2x10⁵, 4x10⁵, 6x10⁵, 8x10⁵, 1x10⁶, 2x10⁶, 4x10⁶, and 6x10⁶ pfu. After the virus infection, cells were rinsed twice with PBS and then incubated with a 0.5% NP-40 and a 3mg/ml ONPG solution. Samples were incubated at room temperature and measured for the formation of ONP every hour for four hours by a spectrophotometer. Development of the assay was monitored until approximate linearity of data was achieved.

Western Blot and Antibody Analysis

Western blot analysis was conducted according to Maniatis *et al.* (1989). Cells were harvested from 60mm dishes and 1ml of 4x SDS-Page buffer (0.576M BME) at 70°C was added to the cells. The cell solution was then boiled for 10 minutes. After 10 minutes 250 μ l of 0.05 iodoacetamide was added to the tube. 125 μ l of the cell solution was added per well of the western gel. The gel was a 10% acrylamide gel composed of a 16X12cm resolving and a 16X4cm stacking section. The gel was run for approximately

16 hours at 10volts. Standards run with the gel were Biorad Kaleidoscope Prestained Standards catalog #161-0324. Protein was transferred electrophoretically to nitrocellulose (Osmonics Inc. Westborough, MA). The transfer was conducted at 500mA for 4 hours in a buffer of 25mM Tris, 192mM glycine, 20% v/v Methanol, at pH 8.3, and proof of transfer was verified using Ponceau S staining post transfer. The blot was probed with Anti-c-myc clone 9E10 from Sigma and then with the secondary antibody anti-mouse IgG specific peroxidase from Sigma. Proteins were visualized using ECL western blotting detection reagents RPN2106 from Amersham Pharmacia Biotech.

Results

Restriction Analysis of Mutant Created by Site-directed Mutagenesis

Figure 3A shows the SacI restriction digest gel for a 1Kb molecular weight standard, pBEC28, and pNW43CG. Restriction analysis of the 1Kb standard run with the samples allowed a plot to be constructed using the log of the molecule weights verses distance migrated (Figure 3B). This allowed fragment sizes for pBEC28 and pNW43CG to be calculated. Plasmid pBEC28 was calculated to have fragment sizes of 4663 and 1196bp. Plasmid pNW43CG was calculated to have fragment sizes of 4663 and 631bp. See attached restriction maps for the predicted locations of SacI restriction sites in pBEC28 and pNW43CG (Figures 1&2).

Western Blot of Cells Transfected with pNW43CG plasmid

Western blot analysis of CHO-K1 cells transfected with pMycHisB, pBEC28, and pNW43CG showed expression of two protein fragments at approximately 32KD by pNW43CG (Figure 4). Expression of proteins by cells transfected with pMycHisB and pBEC28 appeared identical to expression of proteins by pNW43CG except for the absence of 32KD fragments.

Virus Assays of CHO-K1 Cells Expressing pNW43CG mutant HVEM plasmid

The graph of the first virus infection assay done on cells containing pNW43CG is seen Figure 5A. The graph shows that pNW43CG has equivalent β -gal activity/virus mediating ability to the negative control pMycHisB. However, an overall poor transfection for both pMycHisB and pNW43CG was achieved into the CHO-K1 cells. Figure 5B shows the activity of β -gal during the second virus assay. The higher the β -gal activity, the better virus is being mediated into cells. Here pNW43CG has a higher β -gal

activity and thus virus entry mediating ability than pMycHisB. In this assay pBEC28 was also used as a positive control to show the virus mediating ability of normal HVEM. Figure 5B shows that HVEM encoded by pNW43CG has less mediating ability than the HVEM encoded by pBEC28.

Discussion

It is clear from the data presented in this thesis that the original objective, to identify amino acids within CRRD1 of HVEM essential for HVEM-mediated HSV entry, was not achieved. Furthermore, my hypothesis that changing the amino acids within CRRD1 of HVEM would affect virus entry mediation, was not proven. However, data from this thesis have shown that a possible mutation of HVEM does affect its virus entry mediating ability, and a new mutant may have been created during the course of the thesis experiments.

Restriction analysis of the pNW43CG mutant suggests that it differs from the pBEC28 vector. Restriction analysis of pBEC28 resulted in fragments of 4663 and 1196bps, while fragments of 5330, 1132, and 87 were expected. Due to its small size, it is conceivable that the 87bp fragment ran off the gel and was not detected. Fragment sizes of both pBEC28 and pNW43CG were calculated by comparing the migration distances of their fragments to the migration distances of fragments contained in a 1Kb molecular weight marker. This marker did not show clear separation of fragment sizes in the 12000 to 4000Kb range. Therefore, the calculation of the 4663Kb fragment is an approximation at best. Rerunning the fragments through a more concentrated gel or for a shorter period of time would allow for the measurement of the presence or absence of the 87Kb fragment. A different molecular weight marker should also be used so that fragment sizes in the 12000 to 4000Kb range can be more accurately calculated. Restriction analysis of the mutant pNW43CG resulted in fragments of 4663 and 631bps, while fragments of 5330, 674, 458, and 87bps were predicted. The restriction analyses for both pBEC28 and pNW43CG show that pNW43CG has different restriction pattern

from pBEC28. This does not prove that site-directed mutagenesis created a mutant plasmid that encodes HVEM, but it does suggest that the pNW43CG plasmid should be studied further as a possible mutant. Restriction analysis does clearly show that the original desired mutant was not created.

Western blot analysis was used to verify that transfection of the mutant pNW43CG plasmid was successful and that HVEM was being expressed by the CHO-K1 cells. Figure 4 shows a representative western blot. Lane 1 contained pMycHisB, no expression of HVEM was seen, and this was the negative control. Binding in this lane is nonspecific or due to the addition of the anti-myc antibody to the myc tag encoded by the transfected vector. The next lane shows pBEC28, which was predicted to show expression of HVEM encoded by pBEC28. No expression of HVEM is seen in this lane, and this could be due to poor sample preparation or antibody binding. It is believed that pBEC28 was successfully transfected into cells because these cells were successfully infected during the virus viral assays described below. The third lane shows expression of pNW43CG. Expression of proteins around 32KD is clearly indicated by the blot. This may indicate that a form of the HVEM protein was expressed by the transfected CHO-K1 cells.

Two graphs of virus infection assays are shown in Figure 5. Figure 5A does not show that pNW43CG has any different virus mediating ability than the negative control pMycHisB. However, it was believed that the transfection for this experiment was poor. This was indicated by low expression of transfected protein in the western blot for this experiment (data not shown). The experiment was then repeated and in Figure 5B, pNW43CG is shown to have virus entry mediating ability at a higher level than the

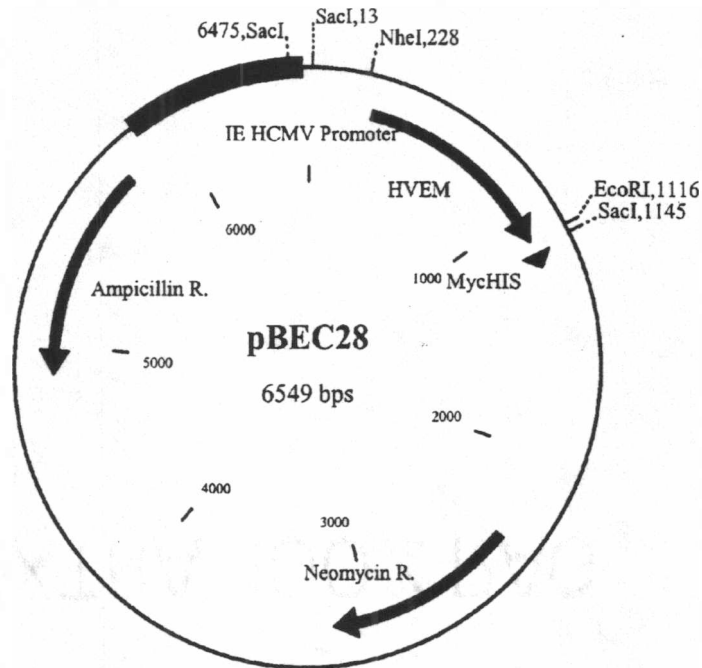
negative control pMycHisB. In this graph the activity of pBEC28 encoding normal HVEM is also shown. A previous study by Shieh *et al.* (1992) showed that CHO-K1 cells are very inefficient at being infected by HSV-1(KOS) virus, and Montgomery *et al.* (1996) showed that virus infection ability of CHO-K1 cells was increased when HVEM was transfected into and expressed by these cells. It is therefore probable that the virus mediating ability described with regard to pBEC28 encoded HVEM is due to proper transfection and expression of HVEM from pBEC28. Figure 5B also shows that the protein encoded by pNW43CG has less virus mediating ability than HVEM from pBEC28. This experiment has been repeated with the same result (personal communication, Montgomery 2002).

Taken together the results of the data from this thesis show three possible conclusions. Site-directed mutagenesis may have produced a mutant form of HVEM encoded by pNW43CG. A mutant protein encoded by pNW43CG seems to be expressible in CHO-K1 cells, and this mutant protein could possibly be a form of HVEM. Finally, the mutant protein encoded by pNW43CG seems to mediate viral entry into CHO-K1 cells at a lower level than normal HVEM encoded by pBEC28.

The original focus of this thesis was to mutate a specific amino acid within HVEM and test this single mutation's ability to affect virus entry mediation. The new focus of this thesis became the characterization of an unexpected mutant that was produced as a result of the mutation procedures. The fact that this mutant showed a virus mediating ability at reduced levels from normal HVEM, warrants further study, which is continuing in Montgomery lab. Determining what in the mutant protein structure causes the reduction in virus entry mediating ability may be important to understanding how

HVEM mediates virus entry at all. This goes right to the heart of the original purpose of this thesis, which was to see how the mutation of different parts of HVEM would affect the virus mediating ability of the HVEM protein.

Figure 1.



Molecule: pBEC28, 6549 bps DNA Circular
 File Name: pBEC28pict.cm5, dated 12 Mar 2002

Description: HVEM cloned into pcdNA3.1 mychisBvector

Notes: Inserted HVEM gene encodes full length HVEM with myc and HIS6 epitope tags on the carboxy terminus of the protein.

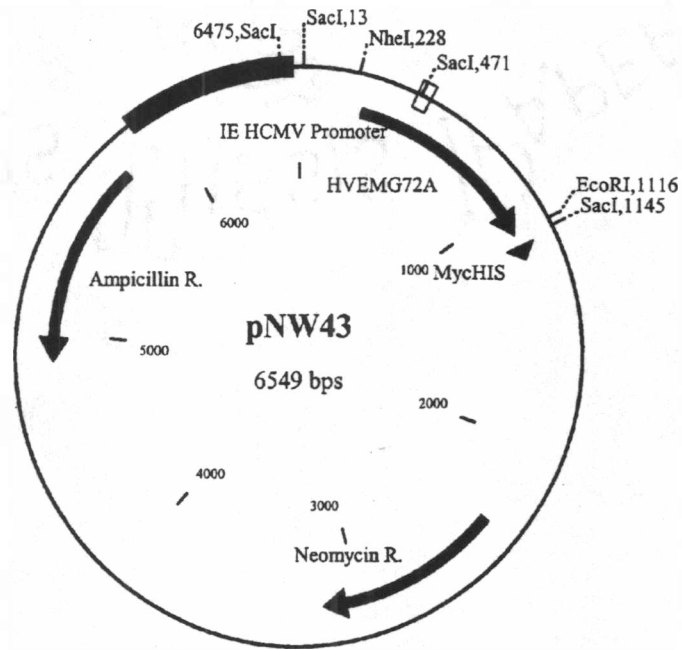
Molecule Features:

Type	Start	End	Name	Description
GENE	267	1115	HVEM	herpes virus entry mediator
GENE	1167	1228	MycHIS	Myc Epitope and PolyHIS tag
GENE	2366	3160	Neomycin R.	G418/Neomycin Resistance Gene
GENE	5747	4886 C	Ampicillin R.	Ampicillin Resistance Gene
REGION	5870	6524	IE HCMV Promoter	Immediate Early Human Cytomegalovirus Promoter

Enzymes (5 sites)

SacI	13,	NheI	228,	EcoRI	1116,	SacI	1145
SacI	6475,						

Figure 2.



Molecule: pNW43, 6549 bps DNA Circular
 File Name: pNW43.cm5, dated 12 Mar 2002
 Description: HVEM-(G72A) introduced SacI at 471
 Notes:

Molecule Features:

Type	Start	End	Name	Description
GENE	267	1115	HVEMG72A	herpes virus entry mediator G72A
MARKER	481			
GENE	1167	1228	MycHis	Myc Epitope and PolyHis Tag
GENE	2366	3160	Neomycin R.	G418/Neomycin Resistance Gene
GENE	5747	4886 C	Ampicillin R.	Ampicillin Resistance Gene
REGION	5870	6524	IE HCMV Promoter	Immediate Early Human Cytomegalo Virus Promoter

Enzymes (6 sites)

SacI	13,	NheI	228,	SacI	471,	EcoRI	1116
SacI	1145,	SacI	6475,				

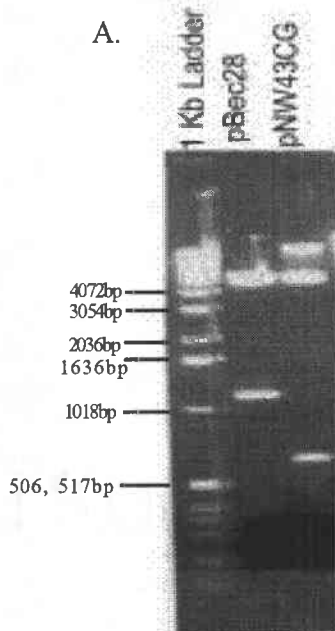
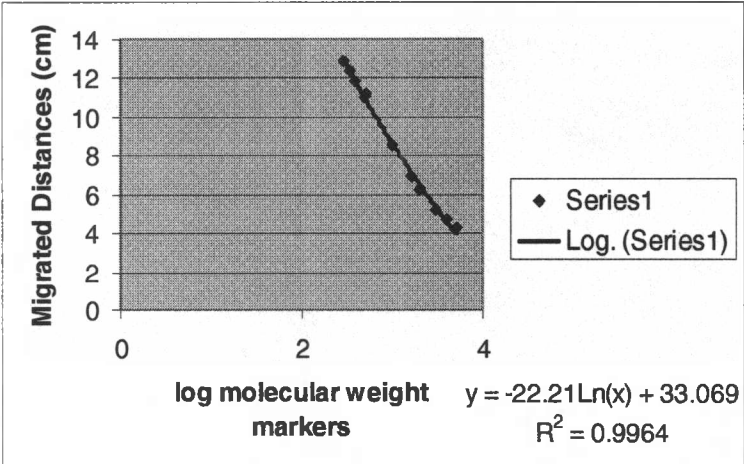


Figure 3: Restriction Analysis of Mutant pNW43CG constructed during Site-directed Mutagenesis.

A. The 1% Agar TBS gel stained with Ethidium Bromide shows the restriction pattern for pBEC28(original mammalian expression vector) versus the restriction pattern for the newly created pNW43CG. In this gel two bands are visible for both pBEC28 and pNW43CG. pBEC28 has bands at 4663bp and 1196bp. pNW43CG has bands at 4663bp and 631bp.

B. Graph showing log of the molecular weight makers for the 1 KB ladder in 3A versus migrated distance. A log regression was used to produce an equation to calculate band sizes for pBEC28 and pNW43CG.

B.



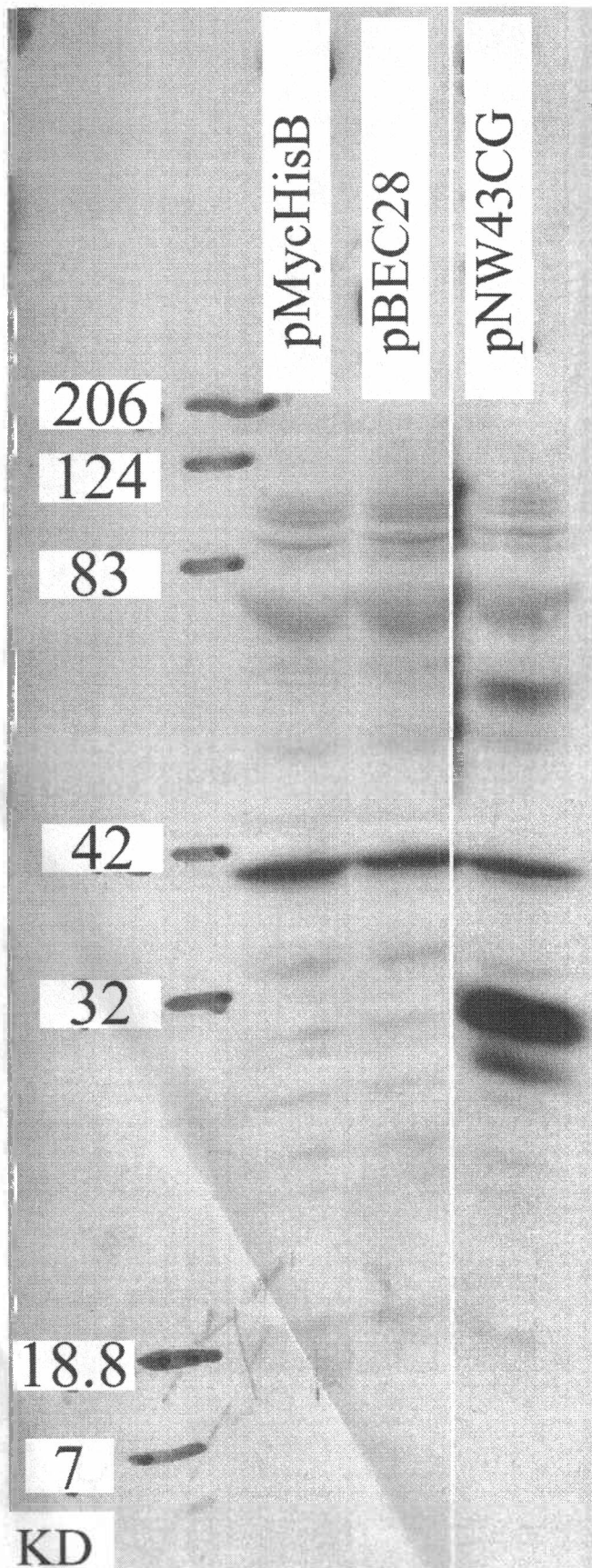


Figure 4. Expression of Mutant HVEM pNW43CG in CHO-K1 Cells.

Western blot showing expression of CHO-K1 cells transfected with plasmids carrying pMycHisB, pBEC28, and pNW43CG. The blot was probed with Anti-c-myc clone 9E10 from Sigma and then the secondary antibody Anti-mouse IgG specific peroxidase from Sigma for R140 primary. Proteins were visualized using ECL Western Blotting Detection Reagents RPN2106 by Amersham Pharmacia Biotech. The blot shows that pNW43CG has two protein fragments around 32 KB that are not seen in pBEC28.

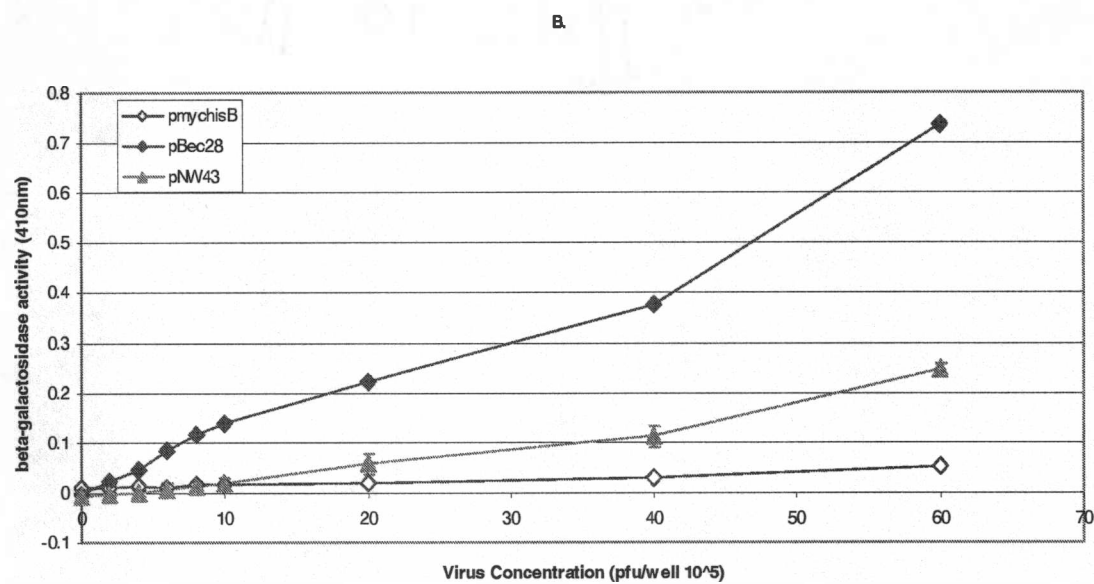
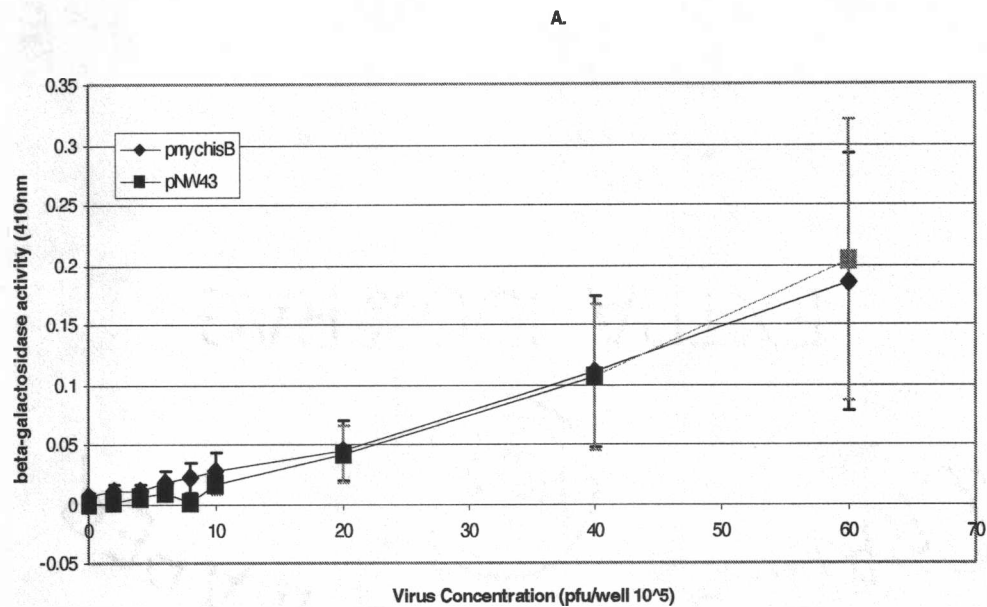


Figure 5. Infection of transiently-transfected CHO-K1 cells with HSV-1(KOS)gL86.
 A. This is a graph of virus infection/ beta-galactosidase activity 300 minutes post addition of ONPG at 410nm. The black diamond line represents activity of pMycHisB and the square gray line represents activity of pNW43CG. Standard Error Bars are present. There is no discernable difference in virus infection/beta-galactosidase activity.
 B. This is a graph of virus infection/ beta-galactosidase activity 300 minutes post addition of ONPG 410nm. The open diamond line represents activity of pMycHisB, the black diamond line represents activity of pBEC28, and the gray triangle line represents activity of pNW43CG. Standard Error Bars are present. pNW43CG shows less beta-galactosidase activity than pBEC28 but much more than pMycHisB.

Table 1. PCR Reaction Reagents

20ng pDNA (pBEC28)
1ul 100nm of each primer
5ul 10x thermopol buffer (1x)
1ul 100x BSA (2x)
5ul 10 mmole dNTPs
1ul vent (2units)
Up to 50ul w/H₂O

Table 2. PCR Program

1. 1min 85°C
 2. 1min 98°C
 3. 1min 55°C
 4. 13min 72°C
- REPEAT STEPS 2-4 17X**
5. 10min 72°C
 6. 4°C overnight storage
-

Literature Cited

- Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., and Berger, E.A. (1996). CC CKR5: A RANTES, MIPdeleted.1a, MIP-1b receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955–1958.
- Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The b-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135–1148.
- Dalglish, A.G., Beverley, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312, 763–766.
- Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., and Landau, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661–666.
- Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., and Doms, R.W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the b-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85, 1149–1158.
- Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagasments, hima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., and Paxton, W.A. (1996). HIV-1 entry into CD41 cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381, 667–673.

- Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872-877.
- Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Spear, P. G. 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* 280, 1610-1620.
- Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J.-C., and Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* 312, 767-768.
- Maddon, P.J., McDougal, J.S., Clapham, P.R., Dalglish, A.G., Jamal, S., Weiss, R.A., and Axel, R. (1988). HIV infection does not require endocytosis of its receptor, CD4. *Cell* 54, 865-874.
- Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87, 427-436.
- Parker J. 2000. Chapter 8 Viruses. In: Corey, P. F., editor. *Brock Biology of Microorganisms*. 9th ed. Upper Saddle River, NJ: Prentice-Hall; 2000. p 236-288.
- Sambrook J., Fritsch E. F., and Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Plainview, NY.
- Shieh, M.-T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* 116, 1273-1281.

Spear, P. G., Eisenberg, R. J., Cohen, G. H. 2000. Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* 275, 1-8.

Wickham, T.J., Mathias, P., Cheresch, D.A., and Nemerow, G.R. (1993). Integrins avb3 and avb5 promote adenovirus internalization but not virus attachment. *Cell* 73, 309-319.

Wilson, N.T. and Montgomery, R. I. 2002. Unpublished data.

Wittels, M., and P. G. Spear. 1991. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. *Virus Res.* 18, 271-290.