Equus caballus Major Histocompatibility Complex Class I Is an Entry Receptor for Equine Herpesvirus Type 1

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In this study, Equus caballus major histocompatibility complex class I (MHC-I) was identified as a cellular entry receptor for the alphaherpesvirus equine herpesvirus type 1 (EHV-1). This novel EHV-1 receptor was discovered using a cDNA library from equine macrophages. cDNAs from this EHV-1-susceptible cell type were inserted into EHV-1-resistant B78H1 murine melanoma cells, these cells were infected with an EHV-1 lacZ reporter virus, and cells that supported virus infection were identified by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. Positive cells were subjected to several rounds of purification to obtain homogeneous cell populations that were shown to be uniformly infected with EHV-1. cDNAs from these cell populations were amplified by PCR and then sequenced. The sequence data revealed that the EHV-1-susceptible cells had acquired an E. caballus MHC-I cDNA. Cell surface expression of this receptor was verified by confocal immunofluorescence microscopy. The MHC-I cDNA was cloned into a mammalian expression vector, and stable B78H1 cell lines were generated that express this receptor. These cell lines were susceptible to EHV-1 infection while the parental B78H1 cells remained resistant to infection. In addition, EHV-1 infection of the B78H1 MHC-I-expressing cell lines was inhibited in a dose-dependent manner by an anti-MHC-I antibody.

MATERIALS AND METHODS

Cells, viruses, antibodies, plasmids, and cDNA library. B78H1 murine melanoma cells were generously provided by Gary Cohen and Roselyn Eisenberg (University of Pennsylvania). B78H1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). The construction and packaging of the equine macrophage cDNA library into the pFB murine retrovirus (7, 48) were described earlier, and this library was kindly provided by Ron Montelaro (University of Pittsburgh). The EHV-1 lacZ reporter virus, L11AgIgE was described previously (16). The monoclonal anti-equine MHC-I antibody C21 was provided by Douglas Antczak (Cornell University). The polyclonal anti-MHC-I antibody HLA B27 (ab93150) was purchased from Abcam (Cambridge, MA). Secondary goat anti-rabbit IgG-horseradish peroxidase (HRP) (sc-2030) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary goat anti-mouse Alexa Fluor 594 antibody was purchased from Invitrogen (Carlsbad, CA). The PCR-amplified equine cDNA (MHC-I clone B9) obtained from the screening process was subcloned into the pCDNA2.1 Topo Vector (Invitrogen) to generate pCDNA2.1TOPO-B9MHC-I. This vector was then digested with MluI and BglII to release the B9 MHC-I gene. The MHC-I gene was ligated into the pCDNA3.1-hygro plasmid (Invitrogen) to generate the MHC-I expression plasmid, pCDNA3.1-B9.

Transduction of the equine cDNA library into B78H1 cells and screening for EHV-1 infectivity. Two 35-mm dishes of B78H1 cells (1 × 10⁷) were seeded. One dish was infected with the pFB murine retrovirus containing the equine macrophage cDNA library (48) at a multiplicity of infection (MOI) of 5, and the other dish was not infected. Forty-eight hours postinfection (p.i.), medium was replaced with DMEM containing 5% FBS. Cells from each dish were harvested,

Equine herpesvirus type 1 (EHV-1) is a major pathogen affecting horses worldwide. Clinical signs of infection range from initial respiratory distress, fever, inappetence, and malaise to more serious secondary conditions including paralysis in some cases and abortigenic disease in pregnant mares (2). The virus is readily spread via direct transmission from horse to horse or via contact with contaminated surroundings. Due to the latent program of the virus, there is a constant reservoir of EHV-1 within the equine population, and frequent reactivation events trigger outbreaks and expose naive horses to the virus (35).

At the cellular level, EHV-1 initially attaches to cells via an interaction between two of its glycoproteins, gC and gB, and cell surface heparin sulfate (36, 41). While these electrostatic interactions mediate virus binding, they do not trigger the entry of the virus into cells. For entry to proceed, a secondary triggering event mediated by gD must occur (10, 14). After fusion between the viral envelope and a cellular membrane, viral capsids are released into the cytoplasm and then actively translocated to the nucleus along microtubules (18).

Previous studies showed that EHV-1 utilizes a cell receptor that is distinct from any of the known alphaherpesvirus entry receptors (14). The goal of the present study was to identify a functional EHV-1 entry receptor by screening an equine macrophage cDNA library. To identify a receptor, we transferred equine cDNAs (48) from an equine macrophage library into cells that are highly resistant to EHV-1. These cDNA-transduced cells were then screened for their ability to mediate EHV-1 infection. Using this approach, we successfully converted a set of highly resistant cells to a state of complete susceptibility to EHV-1. From this converted set of cells, we amplified and sequenced the incorporated equine cDNA. The sequencing results revealed that the equine cDNA isolated from our screen codes for Equus caballus major histocompatibility complex class I (MHC-I) protein, and further assays confirmed that this receptor is utilized by EHV-1 for entry into cells.

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resuspended, and seeded into 96-well plates 24 h after medium replacement, and
the cells were incubated in 5% CO2 at 37°C for 48 h. Duplicate 96-well plates
were prepared for each original 96-well plate, and the cells were allowed to
divide for an additional 48 h. Cells in each original 96-well plate (pFB infected
and mock infected) were infected with EHV-1 L11agLaG at an MOI of 10
for 16 to 20 h and then fixed with 0.5% glutaraldehyde in phosphate-buffered saline
(PBS). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to
the plate, and the wells were screened for the appearance of blue cells. Wells
with the most blue cells were identified, and cells from the corresponding well of
the duplicate plate were trypsinized and subdivided into a 96-well plate (one
96-well plate for each individual well that was selected for further characteriza-
tion). This screening process was repeated for a total of five rounds. After the
final round, cells from individual positive wells were harvested, counted, and then
subdivided into numerous 96-well plates such that each well of the 96-well plate
received approximately a single cell from the harvested cell population. Cells
in these plates were grown for 7 days until they reached confluence. Cells were
then trypsinized and split equally into duplicate plates. After a further 48 h of cell
growth, cells were infected with L11agLaG at an MOI of 3 for 2 h. Cells were
then fixed and stained as described above, and wells that contained 100% blue
cells were isolated from their corresponding duplicate well and used for genomic
data isolation.

Genomic DNA isolation and PCR amplification of equine cDNA. Genomic DNA
(1 × 10^6 cells/sample) was collected from the B78H1 clones (B78H1R+) that
exhibited 100% infection using a Promega Wizard Genomic DNA purification
kit as per the manufacturer’s directions (Promega, Madison, WI). Total DNA
isolated from each sample was quantitated using a NanoVue spectrophot-
ometer (GE Healthcare, Piscataway, NJ), and PCR was performed on each sample
using the following primers: forward 5′-GGCAGGCTTATGCAACCCAGAGAT-3′
and reverse 5′-GCCGGCGTTTATCGAACCCCAGAGAT-3′. DNA was denatured for
5 min at 95°C, and then 35 cycles were run as follows: 15 s at 94°C, 30 s at 55°C,
and 2 min at 72°C, followed by a final extension of 7 min at 72°C. PCR products
were run on a 1% agarose gel and isolated using a Quick-Clean 5 M gel extraction kit (GeneScript Corporation, Piscataway, NJ) following the manufacturer’s protocol.

Detection of PCR products. The PCR products were sequenced by Macrogen, Inc. (Seoul, South Korea), using the forward PCR primer listed above. All sequence data were analyzed using Sequencher, version 4.8, software
(Gene Codes Corporation, Ann Arbor, MI).

Generation of MHC-I receptor lines in B78H1 cells. B78H1 cells were trans-
duced with the pCDNA3.1-B9 MHC-I expression plasmid using Lipofectamine
2000 (Invitrogen) per the manufacturer’s instructions. Transfected cells were
grown in the presence of 100 μg/ml hygromycin B. Hygromycin-resistant cells
were maintained in hygromycin B and then split into a 96-well plate such that
each well was seeded with approximately one cell. After cells reached confluence
in the 96-well plate, they were trypsinized and placed into wells of a 24-well plate.
Cells were grown until confluence and split into a duplicate 24-well plate, and
then one of the plates was infected with L11agLaG at an MOI of 10 for 24 h. The
cells that were not infected with 0.5% glutaraldehyde in PBS, stained with X-Gal,
and screened for the appearance of blue cells. Cells that showed 100% infection
by X-Gal staining were selected for further growth in six-well plates.

Immunofluorescence analysis of MHC-I receptor expression. A total of 5 × 10^6
parental EHV-1-resistant B78H1 cells and the cDNA-transduced B78H1+ cells
selected for their susceptibility to EHV-1 infection were seeded onto number
1.5 coverglass, 35-mm MatTek dishes (MatTek Corporation, Ashland, MA)
and incubated overnight at 37°C in 5% CO2. Cells were washed once with cold
PBS and then fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C. PFA
was removed, and cells were washed with PBS twice for 5 min per wash.
Cells were blocked with 0.1 mg/ml bovine serum albumin (BSA) for 1 h at room
temperature with gentle agitation on a rocking platform. Cells were incubated with
X-Gal and then the separated proteins were transferred to an Immobilon-P polyvinylidi-
dene difluoride (PVDF) membrane (Millipore, Bedford, MA) overnight at 30°C.
The membrane was incubated in 10% nonfat dry milk in Tris-buffered saline-
Tween (TBST) for 1 h at room temperature, and then the rabbit polyclonal
primary anti-MHC-I antibody, MLA B27 (ab93150; Abcam), was added at a concentration of 1:250 for 24 h at 4°C in 5% nonfat dry milk in TBST. The
membrane was washed three times for 10 min each at room tempera-
ture, and then the secondary antibody, goat anti-rabbit IgG-HRP (Santa Cruz
Biotechnology), was incubated on the membrane for 1 h at RT in TBST at a con-
tentration of 1:1,000. The membrane was washed three times for 10 min each
time TBST at room temperature, and then SuperSignal West Pico chemilumines-
cent reagent (Pierce) was added to the membrane for 5 min. The membrane was
exposed to X-ray film and processed.

Semiquantitative reverse transcription-PCR (RT-PCR). A total of 1 × 10^6
B78H1 or B78H1-MHC-I cells were seeded in 60-mm dishes. At 24 h postseed-
ing, total RNA was extracted using an EZNA kit (Omega Bio-Tek, Norcross,
GA) as per the manufacturer’s instructions. mRNA was reverse-transcribed into
cDNA using the avian myeloblastosis virus (AMV) reverse transcriptase and a
first-strand cDNA synthesis kit (Invitrogen). MHC-I cDNA was amplified using equine MHC-specific forward (5′-ATTTGGGAGCGGAAACACCGGG-3′) and
reverse (5′-CCCCATCGTCAGCACCGCA-3′) primers. PCR was run as follows:
initial denaturation for 5 min at 95°C and then 35 cycles consisting of 15 s
at 94°C, 30 s at 55°C, and 2 min at 72°C, followed by a final extension of 7 min
at 72°C. PCR products were run on a 1% agarose gel.

Antibody blocking. A B78H1-MHC-I cells, a total of 2.5 × 10^6 B78H1-MHC-I
cells were seeded in a 96-well plate. Twofold dilutions were made of mouse
anti-equine MHC-I antibody (CZ3). Before antibody was added to the cells, the
maintenance medium was removed, 100 μl of cold DMEM with 10% FBS was
added to each well, and the plate was placed on ice for 5 min. The DMEM and
25 μl of each antibody dilution were added to the cells in triplicate. The cells
were kept on ice for 1 h. The cells were then infected with L11agLaG at an MOI
of 3 for 24 h. The cells were washed with PBS, and 150 μl of ONPG (o-
nitrophenyl-β-D-galactopyranoside) was added to each well. The plate was in-
cubated at 37°C for 30 min. The plate was then read on a plate reader at 405 nm.

RESULTS

Transduction of an equine macrophage cDNA library into
EHV-1-resistant cells and screening for cells that become suscept-
ible to EHV-1. To identify an EHV-1 entry receptor, EHV-
1-resistant B78H1 murine melanoma cells (1 × 10^6 cells) were
infected with a pFB murine retrovirus that contains an equine
macrophage cDNA library (48). The infected cells were
grown for 3 days and then collected and seeded into two
96-well plates. Once the cells reached confluence, they were
trypsinized and split evenly into two plates each in order to
generate duplicate 96-well plates. Cells in each of the dup-
licate plates were infected with the EHV-1 lacZ reporter
virus, L11agLaG (16), at an MOI of 10. In addition, B78H1
cells that were not transduced with the cDNA library were
also infected and served as a negative control.

As shown in Fig. 1, a subset of B78H1 cells that were trans-
duced with the cDNA library stained positive for infection,
while very few blue cells were observed in the nontransduced,
B78H1 control cells (Fig. 1). Eight wells that contained
the highest number of blue cells were selected for further charac-
terization. Cells from the duplicate wells were trypsinized and
split into two 96-well plates, grown for 48 h, and then infected
with L11agLaG. Four wells were selected from this round of
screening, and cells from the corresponding duplicate wells
were isolated and seeded into two more 96-well plates for
additional screening. This process was continued for four more
rounds, and, in the final screening round, three wells were

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selected, and the cells were counted and subdivided into 96-well plates so that each well contained approximately one cell. The cells were then allowed to divide and grow for 7 days, at which time they were trypsinized and split evenly into duplicate plates. After an additional 48 h, the cells were infected with L11ΔgΔgE at an MOI of 3 and then screened for lacZ expression the following day. After this infection, some wells exhibited 100% staining for X-Gal (Fig. 2), indicating that the entire clonal population of cells had become susceptible to EHV-1 infection.

Genomic DNA isolation, PCR amplification, and sequencing of cDNAs from the positive cells. Cells from wells that exhibited 100% X-Gal staining were selected for further characterization. Cells from each selected well were expanded into six-well plates, and then genomic DNA was isolated from each population as described in Materials and Methods. PCR was performed on each genomic DNA isolate using primers that are specific for the flanking regions of the cDNA insert in the retrovirus construct. As shown in Fig. 3, PCR products were obtained from the genomic DNA of the selected EHV-1 susceptible B78H1 cells but not from the parental EHV-1-resistant B78H1 control cells (Fig. 3). The sizes of the PCR products were identical in each sample, with a strong band at approximately 1.5 kb. The 1.5-kb band was isolated and purified from four of the samples, A4, B9, C2, and D4, as described in Materials and Methods. The purified PCR products were sequenced, and the sequence data for each isolated PCR product revealed an exact match for the E. caballus MHC-I antigen (NCBI reference sequence, XM_001915759.1) which was derived from shotgun sequencing of the equine genome (NCBI, EquCab2 chrUn0268). The complete nucleotide and amino acid sequences for this MHC-I receptor are shown in Fig. 4.

Detection of equine MHC-I on B78H1 receptor-positive cells (B78H1R+). The cell surface expression of MHC-I on the cDNA-transduced B78H1 cells (B78H1R+) was assessed by confocal immunofluorescence microscopy. Parental B78H1

FIG. 1. B78H1 cells were mock infected (A) or infected with the retrovirus cDNA library (B), and then each group of cells was subdivided into 96-well plates and infected with EHV-1 L11ΔgΔgE at an MOI of 3. At 16 to 20 h postinfection, cells were stained with X-Gal to allow for identification of EHV-1-infected cells.

FIG. 2. L11ΔgΔgE infection (MOI of 3) of a clonal population of cDNA-transduced B78H1 (A) and parental B78H1 (B) cells. Cells were infected for 20 h and then stained with X-Gal.
and B78H1R+ cells were stained with the anti-MHC-I antibody, CZ3 (9), and then imaged with a confocal microscope, as described in Materials and Methods. As shown in Fig. 5, strong cell surface staining for the MHC-I receptor was observed on the B78H1R/H11001 cells but not on the parental B78H1 cells (Fig. 5). The intense staining was located almost exclusively on the plasma membrane and was observed on all B78H1R+ cells in the monolayer. These results show that the MHC-I receptor is highly expressed on the surface of B78H1R+ cells but not on the parent B78H1 cells.

EHV-1 infection of B78H1 cells stably transfected with MHC-I receptor cDNA and antibody blocking of infection. The B9 MHC-I receptor cDNA was cloned into the pCDNA3.1-hygro plasmid, and stable cell lines expressing the MHC-I receptor (B78H1-MHC-I cells) were generated as described in Materials and Methods. The expression of the equine MHC-I receptor in these cells was assessed by both semiquantitative RT-PCR and Western blotting (Fig. 6). MHC-I mRNA and protein were readily detected in the B78H1-MHC-I cells but not in the parental B78H1 cells. The expression of the MHC-I receptor in the B78H1-MHC-I cells correlated with robust EHV-1 infection, as shown in Fig. 7. The B78H1 parental cells remained resistant to EHV-1 infection.

In order to further test the specificity of the EHV-1–MHC-I interaction, an antibody blocking assay was performed using an antibody specific for equine MHC-I (CZ3). As shown in Fig. 8, the CZ3 antibody blocked EHV-1 infection on the B78H1-MHC-I cells in a dose-dependent manner. Infection was com-

FIG. 3. PCR amplification of cDNA inserts from genomic DNA of clonal cDNA-transduced B78H1 cells that are susceptible to EHV-1 infection. Lane 1, DNA ladder; lane 2, PCR product from control ELR1 plasmid; lane 3, PCR on parental B78H1 cells; lanes 4 to 9, PCR products from clonal B78H1-EHV-1-susceptible cells.

FIG. 4. Nucleotide and amino acid sequences of MHC-I encoded from amplified equine cDNA.
pletely inhibited with the anti-MHC-I antibody out to a dilution of 1/32, and infection was inhibited over 50% at a dilution of 1/512. These results confirm that EHV-1 entry into the B78H1-MHC-I cells is mediated by MHC-I.

**DISCUSSION**

This study revealed that MHC-I is a functional entry receptor for EHV-1. B78H1 murine melanoma cells that are highly resistant to EHV-1 were rendered completely susceptible to EHV-1 infection after expression of the cDNA encoding equine MHC-I. The inability of EHV-1 to infect parental B78H1 cells is most likely explained by the lack of MHC-I receptor expression on these cells (8, 33). Likewise, the ability of EHV-1 to infect a diverse array of cells obtained from different species (42) may be explained by the ubiquitous expression of MHC-I receptors on nearly all nucleated cells (5, 25) and the structural homology exhibited between MHC-I molecules of different species (25).

MHC-I genes are classified as either classical or nonclassical genes based on sequence and structural data (40). Compared to the nonclassical MHC-I genes, the classical genes are highly polymorphic, contain a short transmembrane domain, have a long cytoplasmic tail, and are capable of presenting antigens to CD8+ T cells (24, 40). Additionally, classical MHC-I genes are expressed on nearly all nucleated cells, whereas the nonclassical MHC genes are expressed on a more narrow range of cell types (25). Ellis et al. identified four classical MHC-I genes (ECMHCB1-4) and three nonclassical MHC-I genes (ECMHCA1, ECMHCC1, and ECMHCE1) in the horse by sequence analyses (12). The consensus sequences of the four classical MHC-I genes are grouped together as MHCX1 (accession number NM_001099766).

MHC-I molecules are critically important in host defense against various pathogens. The MHC-I molecules are comprised of a heavy chain bound to β-2-microglobulin, and antigens from intracellular pathogens are processed and loaded onto the MHC complexes in the endoplasmic reticulum by the transporter associated with antigen processing (TAP). The entire antigen–MHC-I complex is then transported and expressed on the surface (28), and recognition of the antigen–MHC-I complex by specific CD8+ T cells triggers cytolysis of the antigen-presenting cells. The ability of antigen-presenting cells to process and present antigens to the cytolytic CD8+ T cells is a critical mechanism involved in the eradication of intracellular pathogens.

Viruses have evolved myriad strategies to counteract this important host cell defense mechanism. Some of the viral immune evasion mechanisms utilized by viruses include the interruption of antigen loading by TAP onto the MHC complex (1, 22, 29, 31), endocytosis of MHC-I (39), and blocking the transport of MHC molecules to the cell surface (21, 27, 32, 38, 46, 47). Many herpesviruses, including the alphaherpesvi-
ruses herpes simplex virus (HSV) (19), bovine herpesvirus type 1 (BHV-1) (20, 23, 30), pseudorabies virus (PRV) (4, 13), and Marek’s disease virus (MDV), (26) downregulate the expression of MHC-I molecules. Immune evasion mechanisms employed by EHV-1 have also been identified. Similar to other alphaherpesviruses, EHV-1 disrupts the ability of TAP to load antigens onto MHC-I molecules. Immune evasion mechanisms mediated by the UL49.5 protein that is highly conserved within the Alphaherpesvirinae (11). In addition to interfering with TAP, EHV-1 has also been shown to decrease overall MHC-I expression on the cell surface at very early times postinfection (3, 37, 43, 44). Rappocciolo et al. concluded from their study that the downregulation of MHC-I is most likely mediated by an EHV-1 early gene. The finding from the present study showing that EHV-1 can utilize MHC-I as a receptor suggests a possible additional viral mechanism for the early downregulation of MHC-I. One possible explanation for the loss of surface MHC-I receptor expression is that the receptor may be internalized together with EHV-1 after EHV-1 engages the receptor. Previous work revealed that EHV-1 enters cells by an endocytic mechanism or by direct fusion at the plasma membrane (17). In addition, HSV-1 has been shown to utilize an endocytic mechanism to gain entry into B78H1 cells engineered to constitutively express the HSV-1 entry receptor nectin-1 (34).

Alternatively, EHV-1 may use MHC-I as a receptor and not internalize this receptor. In this case, EHV-1 could use the receptor to gain entry, but then through its expression of UL49.5, EHV-1 could quickly shut down MHC expression on the cell surface, effectively hiding the virus from circulating

**FIG. 7.** EHV-1 infection of B78H1-MHC-I cell lines. B78H1 (A) and B78H1-MHC-I (B) cells were infected with EHV-1 L11ΔgIΔgE at an MOI of 5 for 20 h and then stained with X-Gal.

**FIG. 8.** The MHC-I antibody CZ3 inhibits EHV-1 infection of B78H1-MHC-I cells in a dose-dependent manner. Cells were incubated for 1 h at 4°C with CZ3 antibody (Ab) at the dilutions indicated on the x axis and then infected with EHV-1 (L11ΔgIΔgE) at an MOI of 3 for 20 h. ONPG substrate was added to the cells, and β-galactosidase activity was assessed by measuring the absorbance at 405 nm.
CD8$^+$ T cells. Current studies are under way to address whether the MHC-I receptor is internalized together with EHV-1 or whether MHC receptors are endocytosed at very early times postinfection due to the action of viral proteins such as UL49.5.

Earlier work revealed that EHV-1 can enter cells by at least two distinct mechanisms. In CHO-K1 cells, EHV-1 is endocytosed, and the viral capsid is released into the cytoplasm after two distinct mechanisms. In CHO-K1 cells, EHV-1 is endocytosed such as UL49.5.

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