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**A SINGLE AMINO ACID RESIDUE IN THE A2 DOMAIN OF MAJOR
HISTOCOMPATIBILITY COMPLEX CLASS I IS INVOLVED IN THE EFFICIENCY OF
EQUINE HERPESVIRUS-1 ENTRY**

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Running title: Critical residue for EHV-1 receptor

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Equine herpesvirus-1 (EHV-1), an alphaherpesvirus of the family *Herpesviridae*, causes respiratory disease, abortion, and encephalomyelitis in horses. EHV-1 utilizes equine MHC class I molecules as entry receptors. However, hamster MHC class I molecules on EHV-1-susceptible CHO-K1 cells play no role in EHV-1 entry. To identify the MHC class I molecule region that is responsible for EHV-1 entry, domain exchange and site-directed mutagenesis experiments were performed, in which parts of the extracellular region of hamster MHC class I (clone C5) were replaced with corresponding sequences from equine MHC class I (clone A68). Substitution of alanine for glutamine at position 173 (Q173A) within the $\alpha 2$ domain of the MHC class I molecule enabled hamster MHC class I C5 to mediate EHV-1 entry into cells. Conversely, substitution of glutamine for alanine at position 173 (A173Q) in equine MHC class I A68 resulted in loss of EHV-1 receptor function. Equine MHC class I clone 3.4, which possesses threonine at position 173, was unable to act as an EHV-1 receptor. Substitution of alanine for threonine at position 173 (T173A) enabled MHC class I 3.4 to mediate EHV-1 entry into cells. These results suggest that the amino acid residue at position 173 of the MHC class I molecule is involved in the efficiency of EHV-1 entry.

Equine herpesvirus-1 (EHV-1), an alphaherpesvirus of family *Herpesviridae*, is the causative agent of respiratory disease, abortion, and a serious neurologic disease known as encephalomyelitis in horses. Currently available vaccines are insufficient to protect against neurological disorders and abortions caused by EHV-1 (1). Outbreaks of EHV-1 encephalomyelitis can devastate farms, riding schools, and veterinary hospitals (2-4). Therefore, EHV-1 is one of the most important pathogens in the equine industry.

EHV-1 attaches to heparan sulfate on the cell surface (5,6). Viral entry into cells occurs through either direct fusion at the plasma membrane or endocytosis (7,8). Among the viral glycoproteins that play important roles in EHV-1 entry, glycoprotein D (gD) is essential for viral entry in all cases (9-12). Recently, the equine MHC class I molecule was identified as a host factor involved in the entry process of EHV-1 (13,14). NIH3T3 mouse fibroblast cells and B78H1 mouse melanoma cells are naturally resistant to EHV-1 infection; however, these cell lines become susceptible to EHV-1 infection by the exogenous expression of equine MHC class I (13,14). The equine MHC class I molecule interacts with EHV-1 gD and mediates EHV-1 entry into equine cell types that are naturally susceptible to EHV-1 infection (14).

MHC class I molecules are known primarily for their role in presenting peptides from intracellular antigens to cytotoxic T cells. MHC

class I is polygenic and polymorphic (15). Fifty equine MHC class I genes were identified among 10 different serologically-defined equine MHC class I haplotypes (16), and seven MHC class I loci were transcribed in an MHC homozygous individual (17). Although previous studies have shown that three equine MHC class I molecules can act as EHV-1 receptors (13,14), it is unclear whether all MHC class I molecules are involved in EHV-1 infection.

Chinese hamster ovary (CHO)-K1 cells are known to be susceptible to EHV-1 infection (10). Unlike equine cells, inhibition of the cell-surface expression of MHC class I molecules by β 2-microglobulin knockdown does not reduce the susceptibility of CHO-K1 cells to EHV-1 infection (14). This result suggests that hamster MHC class I has no role in EHV-1 entry into CHO-K1 cells.

In this study, we expressed hamster MHC class I on NIH3T3 cells and confirmed the inability of hamster MHC class I to mediate the cellular entry of EHV-1. To clarify the region of equine MHC class I responsible for EHV-1 entry, we performed domain exchange and site-directed mutagenesis experiments on the hamster MHC class I backbone, in which parts of the extracellular region were replaced with corresponding sequences from equine MHC class I. Results from these experiments revealed an important amino acid residue of MHC class I that mediates the efficiency of EHV-1 entry, as well as an equine MHC class I gene that does not act as a receptor for EHV-1.

EXPERIMENTAL PROCEDURES

Cells and viruses: NIH3T3 and 293T cells were cultured in DMEM containing 10% FBS. The EHV-1 mutant Ab4-GFP contains a GFP expression cassette between open reading frame (ORF) 62 and ORF 63 (18). Stock viruses were cultured in Equine dermis (E. Derm) cells and were titrated by plaque formation assay on rabbit kidney (RK13) cells.

DNA constructs: Equine MHC class I heavy chain clones A68 and B118 were described previously (14). Equine MHC class I heavy chain clone 3.4 (GenBank accession number: XM_001493795) complementary DNA (cDNA) was obtained by RT-PCR from a primary cultured equine brain microvascular endothelial cell cDNA

library (14). Hamster MHC class I heavy chain clone C5 (GenBank accession number: AY064390) cDNA was obtained by RT-PCR from CHO-K1 cells. These cDNA fragments were cloned into the pCXSN vector (14). pCXSN-FLAG-A68, pCXSN-FLAG-B118, and pCXSN-HA-C5 were constructed by insertion of the FLAG or HA epitope tag between the putative signal sequence and the remaining coding sequence.

Equine and hamster MHC class I domain-exchanged mutants were generated with the In-Fusion Advantage PCR Cloning Kit (Clontech Laboratories, Palo Alto, CA) in accordance with the manufacturer's instructions. Amino acid substitutions in MHC class I were introduced by PCR-mediated site-directed mutagenesis.

EHV-1 infection: NIH3T3 cells seeded in 24-well plates were transfected with plasmid DNA encoding MHC class I or its mutant by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 24 h after transfection, cells were infected with Ab4-GFP at a multiplicity of infection (MOI) of 10. At 12 h postinfection (p.i.), GFP-expressing cells were observed under an inverted fluorescence microscope (IX70, Olympus, Tokyo, Japan) and counted by flow cytometry.

Infectivity neutralization assay: For neutralization of viral gD, EHV-1 Ab4-GFP at an MOI of 10 was incubated with anti-gD rabbit polyclonal antibody (generated in the author's laboratory) or control rabbit IgG (Beckman Coulter, Fullerton, CA) for 30 min at 37 °C, and then added to the cells. After incubation for 1 h, extracellular virus was inactivated by treatment with 0.1 M citrate buffer (pH 3.0). The cells were cultured in fresh growth medium for an additional 12 h p.i. Viral entry was assessed by counting the number of GFP-positive cells with flow cytometry.

Ig fusion protein: Soluble gD-Ig fusion protein (gD-Ig) consisting of the extracellular domain of EHV-1 gD and the Fc segment of the human IgG1 was generated as previously described (14). Purified human IgG (Invitrogen) was used as a control (control-Ig).

Flow cytometry analysis: NIH3T3 cells were plated in 12-well plates and transiently transfected with expression plasmids with Lipofectamine 2000 (Invitrogen). 293T cells were plated in 12-well plates and cotransfected with equine MHC class I and equine β 2-microglobulin expression plasmids with TransIT-2020 (Mirus Bio, Madison,

WI). After transfection, cells were stained with primary mouse mAbs against FLAG (clone M2, Sigma, St. Louis, MO), HA (clone HA-7, Sigma), or equine MHC class I (clone H58A, VMRD, Pullman, WA). Bound antibodies were visualized by the addition of Alexa Fluor 488-conjugated anti-mouse IgG antibody (Invitrogen). Flow cytometric analysis was performed with a FACS Canto system (BD Biosciences, San Jose, CA). The data collected were analyzed with the Flowjo software package (Tree Star, Ashland, OR).

To assess the binding of the gD-Ig fusion protein to equine MHC class I, cells were detached and incubated with gD-Ig or control-Ig at 4 °C for 30 min. Bound gD-Ig or control-Ig was stained with phycoerythrin-conjugated anti-human IgG antibody (Beckman Coulter). The fluorescence signal was analyzed by flow cytometry.

Cell-based ELISA: 293T cells plated in poly-L-lysine-coated 48-well plates were cotransfected with the indicated equine MHC class I and equine β 2-microglobulin expression plasmids as described above. After 20 h, cells were incubated with serially diluted gD-Ig or control-Ig in DMEM containing 10% FBS and 20 mM HEPES (pH 7.4) for 30 min at room temperature. The cells were washed with PBS containing 0.1% BSA and fixed with PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde. Fixed cells were exposed to HRP-conjugated anti-human IgG antibody (Jackson ImmunoResearch, West Grove, PA). Signals were developed by adding O-phenylenediamine dihydrochloride substrate (OPD; Sigma) in phosphate-citrate buffer (pH 5.0). After addition of 3N HCl, the absorbance at 490 nm was measured with a microplate reader (BioRad, Hercules, CA).

Immunoprecipitation and immunoblotting: 293T cells were cotransfected with indicated equine MHC class I and equine β 2-microglobulin expression plasmids as described above. After 36 h, cells were lysed in a lysis buffer (1% Brij 98, 200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5% glycerol and complete protease inhibitor cocktail (Roche, Basel, Switzerland)). Lysates were immunoprecipitated with Dynabeads Protein A (Invitrogen) for 2 h at 4 °C after coating with gD-Ig or control-Ig. After washing with lysis buffer, precipitated protein complexes were eluted by boiling in SDS-PAGE sample buffer and separated by SDS-PAGE. Proteins were

transferred onto Immobilon-P transfer membranes (Millipore, Bedford, MA) and detected with anti-DYKDDDDK (FLAG tag) monoclonal antibody (clone L5, BioLegend, San Diego, CA) or anti-human IgG antibody (Jackson ImmunoResearch).

Molecular modeling: A homology model of equine MHC class I A68 was constructed based on the crystal structure of HLA-B (Protein Data Bank accession code: 2BVO). After 100 models were generated by MODELLER 9v6 (19), a model was chosen by a combination of the Modeller objective function value and the discrete optimized protein energy (DOPE) statistical potential score (20). After the addition of hydrogen atoms, the model was refined by energy minimization with the CHARMM force field and the Discovery Studio 2.5 software package (Accelrys, San Diego, CA). Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was ≤ 0.01 kcal/mol/Å. The generalized Born implicit solvent model (21,22) was used to model the effects of solvation. The molecular model was evaluated with PROCHECK (23) and VERIFY-3D (24), and the model structure was displayed by PyMOL (DeLano Scientific LLC) (25).

RESULTS

Expression of the hamster MHC class I molecule does not confer EHV-1 susceptibility to NIH3T3 cells. We obtained the hamster MHC class I heavy chain clone C5 cDNA (26) from CHO-K1 cells. C5 showed 70% amino acid sequence identity and 94% similarity with the equine MHC class I heavy chain clone A68, which we previously identified as an EHV-1 receptor.

NIH3T3 cells are naturally resistant to EHV-1 infection (14); however, exogenous expression of FLAG-tagged A68 rendered the cells susceptible to infection by the GFP-expressing EHV-1 mutant strain Ab4-GFP (Fig. 1A). By contrast, no GFP signal was observed in NIH3T3 cells transfected with HA-tagged C5 (Fig. 1A). Cell-surface expressions of A68 and C5 were confirmed by staining with anti-FLAG and anti-HA tag antibodies, respectively (Fig. 1A). These data suggest that the hamster MHC class I clone C5 expressed on the NIH3T3 cell surface does not mediate EHV-1 entry.

Displacement of $\alpha 2$ domain confers EHV-1 receptor function to the hamster MHC class I molecule. MHC class I heavy chain is a type I transmembrane protein composed of three extracellular domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), a transmembrane domain, and a cytoplasmic tail (27). To identify the domain responsible for EHV-1 entry, we constructed three mutants, C5A68($\alpha 1$), C5A68($\alpha 2$), and C5A68($\alpha 3$), in which each extracellular domain of C5 was exchanged with that of A68 (Fig. 1B). The expression level of the C5A68($\alpha 1$) mutant on the NIH3T3 cell surface was relatively low compared to that of the C5A68($\alpha 2$) and C5A68($\alpha 3$) mutants (Fig. 1C). Among those three mutants, only C5A68($\alpha 2$) supported EHV-1 entry into NIH3T3 cells (Fig. 1C and D).

We then constructed four additional C5 mutants, C5A68(115–161), C5A68(162–206), C5A68(162–187), and C5A68(188–206), in which each segment of the $\alpha 2$ domain was substituted with the corresponding region of A68 (Fig. 1B). Entry of Ab4-GFP occurred in NIH3T3 cells expressing C5A68(162–206) or C5A68(162–187), but not in NIH3T3 cells expressing C5A68(115–161) or C5A68(188–206) (Fig. 1C and D). These results suggest that the amino acid residues at positions 162–187 of A68 play a role in EHV-1 receptor function.

Single-point mutation in the $\alpha 2$ domain confers EHV-1 receptor function to the hamster MHC class I molecule. Between amino acid residues at positions 162 and 186, A68 and C5 differ in four positions (Fig. 2A). To identify the amino acid residues responsible for function of the EHV-1 receptor, we mutated these four positions individually in C5 (Fig. 2A). All of these C5 mutants were expressed on the NIH3T3 cell surface upon transfection; however, EHV-1 entry was observed only in NIH3T3 cells expressing C5 Q173A (Fig 2B and 2C).

We previously demonstrated the involvement of EHV-1 gD in equine MHC class I-mediated EHV-1 entry (14). To examine whether EHV-1 gD is also involved in viral entry via C5 Q173A, we tested the ability of anti-gD polyclonal antibody to block the EHV-1 infection of C5 Q173A-expressing cells. Anti-gD inhibited EHV-1 entry into C5 Q173A-expressing cells in a dose-dependent manner (Fig. 2D). These results

indicate that the hamster MHC class I C5 Q173A mutant acts as a weak receptor for EHV-1.

Single-point mutation in the $\alpha 2$ domain impairs EHV-1 receptor function of equine MHC class I molecules. We next constructed a series of A68 mutants with replacement of amino acid residues at positions between 162 and 186 of A68 with those of C5 (summarized in Fig. 3A), and examined the susceptibility of NIH3T3 cells transfected with these mutants to EHV-1 infection. The W171L mutation reduced cell-surface expression of A68 and inhibited viral entry into NIH3T3 cells (Fig. 3B and C). The A173Q mutation drastically decreased EHV-1 receptor function, although similar levels of cell-surface expression were observed between A68 A173Q and other mutants (Fig. 3B and C). By contrast, EHD178DRQ and N182A did not impair EHV-1 receptor function (Fig. 3B and C).

A previous study showed that another equine MHC class I clone (B118) also acts as an EHV-1 receptor (14). The amino acid sequence of B118 showed high similarity with that of A68 (84% identity, 96% similarity), and the alanine residue at position 170 in B118 was equivalent to the alanine residue at position 173 in A68 (Fig. 4A). Induction of the A170Q mutation in B118 greatly decreased the efficiency of EHV-1 entry, although both B118 and B118 A170Q displayed comparable cell-surface expression levels (Fig. 4B and C). These mutagenesis studies reveal that a single amino acid within the $\alpha 2$ domain of equine MHC class I is important for the efficient entry of EHV-1.

Alanine residue at position 173 of equine MHC class I A68 is required for the interaction of A68 with EHV-1 gD. Because equine MHC class I mediates viral entry via its interaction with EHV-1 gD (14), we investigated the influence of the A173Q mutation on the binding of A68 with EHV-1 gD, using a soluble gD-Ig fusion protein consisting of the extracellular domain of EHV-1 gD and the Fc segment of human IgG1. Flow cytometric analysis showed that the interaction of gD-Ig with A68 A173Q was much weaker than that with A68, although the level of cell-surface expression of A68 A173Q was comparable to that of wild-type A68 (Fig. 5A). Similarly, the A170Q mutation in B118 resulted in a greatly reduced affinity for EHV-1 gD (Fig. 5A).

To further examine the effect of the A173Q mutation on the interaction between A68 and gD, cell-based ELISA and immunoprecipitation assays were performed with A68 or A68 A173Q-expressing cells. Cell-based ELISA showed that gD-Ig bound to A68-expressing cells in a dose-dependent manner (Fig. 5B). By contrast, gD-Ig did not bind to A68 A173Q-expressing cells (Fig. 5B). Interaction of gD-Ig with A68, but not with A68 A173Q, was observed by immunoprecipitation using gD-Ig (Fig. 5C). These results suggest that substitution of a glutamine residue for alanine at position 173 in A68 (or at position 170 in B118) deprives equine MHC class I of gD-binding capacity, which is required for MHC class I to act as an EHV-1 receptor.

Hydrophobicity of the amino acid at residue 173 in equine MHC class I A68 mediates EHV-1 receptor function. Because no crystal structures of equine MHC class I molecules are available, homology modeling of the extracellular domain of the equine MHC class I A68 was performed based on the crystal structure of human MHC class I HLA-B (PDB code: 2BVO). The alanine residue at position 173 was part of an α -helical structure in the $\alpha 2$ domain and was located near the apical surface of equine MHC class I A68 (Fig. 6).

We investigated the effect of mutations at position 173 in A68 to amino acids of various hydrophobicities. These mutants displayed comparable cell-surface expression levels (data not shown). The alanine residue has a hydrophobic side chain. Replacement of the alanine at position 173 in A68 by valine or methionine, which are also hydrophobic, had little effect on the function of the EHV-1 receptor (Fig. 7). Replacement of the alanine at position 173 by glutamine, which is relatively hydrophilic, greatly decreased the EHV-1 receptor function (Fig. 7). Replacement of alanine by more hydrophilic amino acids (A173T, A173S, A173E, and A173N) greatly decreased activity of the EHV-1 receptor (Fig. 7). These results suggest that the hydrophobicity of the amino acid at residue 173 of equine MHC class I may be involved in the ability of MHC class I to act as an EHV-1 receptor.

Equine MHC class I with threonine at position 173 is unable to act as an EHV-1 receptor. Some equine MHC class I genes reportedly have valine, glutamic acid, or threonine instead of alanine at the position corresponding to position 173 of A68

(16). Among the equine MHC class I nucleotide sequences registered in GenBank, we successfully obtained the equine MHC class I clone 3.4 cDNA, which has a threonine residue at position 173, from primary equine cells by RT-PCR (Fig. 8A). NIH3T3 cells transfected with equine MHC class I clone 3.4 were not susceptible to EHV-1 infection, although surface expression on NIH3T3 cells was confirmed by flow cytometry with an anti-equine MHC class I antibody (Fig. 8B and C).

To determine whether the threonine residue at position 173 of clone 3.4 is involved in the inability of this clone to act as an EHV-1 receptor, the T173A mutation was induced in clone 3.4. As expected, NIH3T3 cells expressing equine MHC class I clone 3.4 T173A showed susceptibility to EHV-1 infection (Fig. 8B and C). Furthermore, preincubation of the virus with anti-gD polyclonal antibody reduced EHV-1 entry into equine MHC class I clone 3.4 T173A-expressing cells (Fig. 8D). These data suggest that equine MHC class I clone 3.4, which is expressed naturally on equine cells, is unable to function as an EHV-1 receptor. The findings also suggest that the residue at position 173 of equine MHC class I is involved in the efficiency of EHV-1 entry into cells.

DISCUSSION

The present study revealed that a single amino acid at position 173 of equine MHC class I plays an important role in the entry of EHV-1. Although hamster MHC class I C5 showed amino acid sequence similarity to A68, C5 was unable to mediate EHV-1 entry into cells. A glutamine to alanine substitution at position 173 enabled C5 to act as an EHV-1 receptor, whereas an alanine to glutamine substitution at position 173 in equine MHC class I A68 resulted in loss of the EHV-1 receptor function. The importance of the amino acid residue at position 173 of equine MHC class I is further suggested by the finding that an alanine substitution at position 173 of equine MHC class I clone 3.4 (which does not naturally work as an EHV-1 receptor) resulted in the acquisition of EHV-1 receptor function.

Equine MHC class I reacts with EHV-1 gD, and this interaction mediates EHV-1 entry into cells (14). Soluble gD-Ig fusion protein bound to equine MHC class I A68 and B118 on the cell surface. However, a mutation of alanine at

position 173 in A68 or at position 170 in B118 impaired the interaction of gD-Ig with A68 or B118, respectively. These results indicate that these residues are directly involved in binding to EHV-1 gD, and thereby influence EHV-1 receptor function.

Although EHV-1 infection occurred in NIH3T3 cells expressing hamster MHC class I C5 or equine MHC class I clone 3.4 with an alanine substitution at position 173, the infection rate was lower than that in NIH3T3 cells expressing equine MHC class I clone A68. This finding suggests that other amino acid residues, in addition to alanine 173, contribute to EHV-1 receptor function.

MHC class I molecules are polymorphic and broadly distributed in mammalian bodies (15). Tallmadge et al. (16) cloned 50 equine MHC class I genes from 10 different MHC class I haplotypes and classified their deduced amino acid sequences. Based on the amino acid alignment, 32 of the 50 genes have alanine at the position corresponding to position 173 of A68. In addition to A68 and B118, which we previously identified as an EHV-1 receptor, another equine MHC class I molecule (GenBank accession number: XM_001915759) that reportedly mediates EHV-1 entry has alanine at the position corresponding to position 173 of A68 (13). In this study, we showed that equine MHC class I clone 3.4 is unable to act as an EHV-1 receptor, and further showed that threonine at position 173 is involved in the nonfunction as an

EHV-1 receptor. Although the ability of other equine MHC class I molecules to mediate EHV-1 entry remains unclear, our findings suggest that EHV-1 utilizes a subset of equine MHC class I molecules in viral entry into equine cells.

The extracellular region of MHC class I heavy chain is composed of three domains ($\alpha 1$, $\alpha 2$, and $\alpha 3$), and the $\alpha 1$ and $\alpha 2$ domains lie at the tip of MHC class I (27). Our molecular model suggests that the residue at position 173 within the $\alpha 2$ domain of A68 protrudes outside, as it is accessible to virus. It will be interesting to investigate how the amino acid residue at position 173 contributes to the interaction between MHC class I and EHV-1. Our substitution experiment suggests that the hydrophobicity of the amino acid at residue 173 may influence the efficiency of the MHC class I-dependent entry of EHV-1.

In this study, we identified an amino acid residue in the $\alpha 2$ domain of equine MHC class I as a determinant involved in the efficiency of EHV-1 entry into cells. To further understand the interaction between EHV-1 and equine MHC class I molecules, structural determination of the complex formed by EHV-1 and equine MHC class I will be needed. The current study opens the way towards understanding the interaction between EHV-1 and equine MHC class I molecules at the molecular level, and provides new insights into the host-virus interaction of EHV-1 infection.

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FOOTNOTES

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The abbreviations used are: EHV-1, equine herpesvirus-1; gD, glycoprotein D; IB, immunoblot; IP, immunoprecipitation; MOI, multiplicity of infection; p.i., postinfection.

FIGURE LEGENDS

Fig. 1. EHV-1 entry into NIH3T3 cells expressing equine, hamster, and hamster/equine chimeric MHC class I molecules. (A) NIH3T3 cells were transfected with FLAG-tagged A68 (upper, solid line) or HA-tagged C5 (lower, solid line) expression plasmids. Cell-surface expression of A68 and C5 was assayed by flow cytometry with anti-FLAG and anti-HA antibodies. Dashed line shows mock vector-transfected cells. Transfected cells were infected with the EHV-1 Ab4-GFP strain, and GFP signals were detected by fluorescent microscopy at 12 h p.i. (right panels). Scale bars: 100 μ m. (B) Schematic representation of

hamster/equine chimeric MHC class I constructs. MHC class I was composed of three extracellular domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$), a transmembrane domain (TM), and a cytoplasmic domain (C-tail), as indicated in the upper part of the schema. Solid and dotted lines indicate amino acid sequences corresponding to equine MHC class I A68 and hamster MHC class I C5, respectively. N-terminal FLAG and HA tags are indicated by the box. (C) NIH3T3 cells transfected with the indicated chimeric molecules were analyzed by flow cytometry with anti-HA antibody (left panels, solid line). Mock plasmid-transfected cells were used as a control (left panels, dashed line). Transfected cells were infected with EHV-1 Ab4-GFP (right panels). Scale bars: 100 μm . (D) GFP-positive cells at 12 h after EHV-1 Ab4-GFP infection were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations.

Fig. 2. Effect of mutations in hamster MHC class I C5 on EHV-1 entry. (A) Schematic representation of site-directed mutagenesis of hamster MHC class I C5. All mutants were prepared by replacement of the indicated amino acids of C5 with the corresponding amino acids from the A68 sequence. Dots indicate identical amino acids to C5. Numbers indicate positions of amino acids in the predicted sequence of full-length C5. (B) NIH3T3 cells were transfected with the indicated HA-tagged C5 mutants. At 24 h after transfection, cell-surface expression of the mutants was analyzed by staining with an anti-HA antibody (left panels, solid line). Mock plasmid-transfected cells were used as a control (left panels, dashed line). Transfected cells were infected with EHV-1 Ab4-GFP, and GFP signals were detected by fluorescent microscopy at 12 h p.i. (right panels). Scale bars: 100 μm . (C) GFP-positive cells at 12 h after EHV-1 Ab4-GFP infection were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations. (D) Neutralization assay of EHV-1 gD. EHV-1 Ab4-GFP was incubated with anti-gD antibody (closed circles) or control rabbit IgG (open circles) for 30 min. Each virus-antibody mixture was added to NIH3T3 cells transfected with C5 (left panel) or C5 Q173A (right panel). GFP-positive cells were counted by flow cytometry. Error bars represent standard deviations of three independent samples.

Fig. 3. Effect of mutations in equine MHC class I A68 on EHV-1 entry. (A) Schematic representation of site-directed mutagenesis of equine MHC class I A68. All mutants were made by replacement of the indicated amino acids of A68 with the corresponding amino acids from the C5 sequence. Dots indicate identical amino acids to A68. Numbers indicate positions of amino acids in the predicted sequence of full-length A68. (B) NIH3T3 cells were transfected with the indicated FLAG-tagged A68 mutants. At 24 h after transfection, cell-surface expressions of the mutants were analyzed by flow cytometry with an anti-FLAG antibody (left panels, solid line). Mock plasmid-transfected cells were used as a control (left panels, dashed line). Transfected cells were infected with EHV-1 Ab4-GFP, and GFP signals were detected by fluorescent microscopy at 12 h p.i. (right panels). Scale bars: 100 μm . (C) GFP-positive cells at 12 h after EHV-1 Ab4-GFP infection were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations.

Fig. 4. Effect of a mutation in equine MHC class I clone B118 on EHV-1 entry. (A) Schematic representation of site-directed mutagenesis of equine MHC class I B118. Numbers indicate positions of amino acids in the predicted sequence of full-length A68 and B118. Dots indicate identical amino acids to A68. (B) NIH3T3 cells were transfected with the equine MHC class I B118 or B118 A170Q mutant. At 24 h after transfection, cells were stained with an anti-equine MHC class I antibody (left panels, solid line). Mock plasmid-transfected cells were used as a control (left panels, dashed line). Transfected cells were infected with EHV-1 Ab4-GFP (right panels), and GFP signals were detected by fluorescent microscopy at 12 h p.i. Scale bars: 100 μm . (C) GFP-positive cells at 12 h after EHV-1 Ab4-GFP infection were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations.

Fig. 5. Effect of mutations in equine MHC class I molecules on the interaction with EHV-1 gD. (A) Flow cytometric analysis for the binding of gD-Ig to cell surface equine MHC class I molecules. 293T cells

were cotransfected with the indicated FLAG-tagged equine MHC class I constructs and equine β 2-microglobulin. Cell-surface expression of equine MHC class I was confirmed by staining with an anti-FLAG antibody (left panels, solid line). Cells were treated with gD-Ig (middle panels, solid line) or control-Ig (right panels, solid line) for evaluation of gD binding. Mock plasmid-transfected cells were used as a control (all panels, dashed line). (B) Cell-based ELISA for detection of binding of gD-Ig to equine MHC class I. 293T cells cotransfected as described above were incubated with gD-Ig (left panel) or control-Ig (right panel). Binding was detected by HRP-conjugated anti-human IgG antibody, followed by *o*-phenylenediamine dihydrochloride substrate. Mock plasmid-transfected cells were used as a control. (C) Immunoprecipitation (IP) of equine MHC class I with gD-Ig. Protein A beads coupled with gD-Ig (arrowhead) or control-Ig (open arrowhead) were incubated with cell lysates of 293T cells cotransfected with the indicated FLAG-tagged equine MHC class I constructs (arrow) and equine β 2-microglobulin. Incubated beads and cell lysates were subjected to immunoblot (IB) analyses with anti-human IgG. Lysates from mock plasmid-transfected cells were used as a control.

Fig. 6. Three-dimensional structure of equine MHC class I A68. The crystal structure of HLA-B (PDB code 2BVO) was used as a template for homology modeling. Alanine at position 173 (A173, yellow) is shown as a space-filling model.

Fig. 7. Effect of different amino acid substitutions at position 173 of equine MHC class I A68 on EHV-1 entry. NIH3T3 cells were transfected with the indicated equine MHC class I A68 or A68 mutants. At 24 h after transfection, cells were infected with EHV-1 Ab4-GFP for 12 h. GFP-positive cells were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations.

Fig. 8. EHV-1 infection of NIH3T3 cells expressing equine MHC class I clone 3.4. (A) Schematic representation of equine MHC class I clone 3.4 and its point mutant. Numbers indicate positions of amino acids in the predicted sequence of full-length A68 and clone 3.4. Dots indicate identical amino acids to A68. (B) NIH3T3 cells were transfected with the equine MHC class I 3.4 or 3.4 T173A mutant. At 24 h after transfection, cells were stained with an anti-equine MHC class I antibody (left panels, solid line). Mock plasmid-transfected cells were used as a control (left panels, dashed line). Transfected cells were infected with EHV-1 Ab4-GFP for 12 h and analyzed by fluorescent microscopy (middle and right panels). Scale bars: 100 μ m. (C) GFP-positive cells at 12 h p.i. with EHV-1 Ab4-GFP were counted by flow cytometry. Bars show the means from three samples, and error bars show standard deviations. (D) Neutralization assay of EHV-1 gD. EHV-1 Ab4-GFP was incubated with anti-gD antibody (closed circles) or control rabbit IgG (open circles) for 30 min. Each virus-antibody mixture was added to NIH3T3 cells transfected with equine MHC class I clone 3.4 (left panel) or clone 3.4 T173A mutant (right panel). GFP-positive cells were counted by flow cytometry. Error bars represent standard deviations of three independent samples.