The Lipid Raft-Associated Protein CD98 is Required for Vaccinia Virus Endocytosis

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Running title: Role of CD98 in vaccinia virus entry

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Abstract

Vaccinia mature virus (MV) infects a broad range of animals in vivo and cell cultures in vitro; however, the cellular receptors that determine vaccinia MV tropism and entry pathways are poorly characterized. Here, we performed quantitative proteomic analyses of lipid raft-associated proteins upon vaccinia MV entry into HeLa cells. We found that a type II membrane glycoprotein, CD98, is enriched in lipid rafts upon vaccinia MV infection when compared to mock-infected HeLa cells. Knockdown of CD98 expression in HeLa cells significantly reduced vaccinia MV entry. Furthermore, CD98 knockout (KO) mouse embryonic fibroblasts (MEFs) also exhibited reduced vaccinia MV infectivity without affecting MV attachment to cells, suggesting a role for CD98 in the post-binding step of virus entry. Further characterization with inhibitors and dominant negative proteins that block different endocytic pathways revealed that vaccinia MV entry into MEFs occurs through a clathrin-independent, caveolin-independent, dynamin-dependent fluid-phase endocytic pathway, implying that CD98 plays a specific role in the vaccinia MV endocytic pathway. Infection of wild-type and CD98 KO MEF cells with different strains of vaccinia MV provided further evidence that CD98 plays a specific role in MV endocytosis, but not in plasma membrane fusion. Finally, different CD98-C69 chimeric proteins were expressed in CD98 KO MEF; but none were able to reconstitute MV infectivity, suggesting that the overall structure of the CD98 protein is required for vaccinia MV endocytosis.
Introduction

Vaccinia virus is the prototype of orthopoxvirus genus Poxviridae that includes variola virus, the causative agent of smallpox disease. Vaccinia virus is a large enveloped double-stranded DNA virus and replicates in the cytoplasm of the host cell. It has a wide host range and infects many cell cultures in vitro and animal species in vivo (39). Virus-infected cells produce multiple forms of the infectious particles, of which the mature virus (MV) constitutes the majority. While most MV particles are released from the cells upon lysis, a proportion of MV acquires additional membranes from the Golgi apparatus and is exocytosed to form the extracellular virus (EV) (15).

Vaccinia MV entry pathways and the virus-induced signaling are complex in nature and dependent on the viral strains (4, 36) and the host cell types (16, 36, 50, 62). MV binds to cell-surface glycosaminoglycans (GAGs) (13, 25, 35) and the extracellular matrix protein laminin (12) and subsequently enters cells through either endocytosis (24, 54) or plasma membrane fusion (2, 7, 8, 17, 36, 55). Analyses of vaccinia mutant viruses revealed that two envelope proteins A25/A26 control virus entry pathway specificity, i.e. MV containing A25/A26 proteins are endocytosed into HeLa cells whereas MV particles with no A25/A26 proteins enter cells through plasma membrane fusion (9). The MV endocytic route in HeLa cells was reported as dynamin-independent macropinocytosis (38) and as dynamin- and VPEF-dependent fluid-phase endocytosis (26). So far, however, cell surface membrane proteins necessary for virus binding and penetration have not been reported.

The type II membrane glycoprotein CD98, also known as 4F2 or SLC3A2, is expressed in all cell types with the exception of platelets (49, 60). CD98 constitutes the heavy chain of the family of the heterodimeric amino acid transporters (HATs) (10, 59). So far, six light subunits have been shown to associate with CD98, these include LAT1, LAT2, y+LAT1,
y+LAT2, asc1, xCT and these confer specific amino acid transport activity to the heteromeric complex (58). In addition, CD98 has been demonstrated to interact with certain integrin β-subunits to promote integrin-dependent signaling leading to the activation of FAK, PI3K, Akt, Rac, and the adhesion molecule p130Cas (20, 22, 63).

Our previous study showed that vaccinia MV clustered at the lipid rafts of plasma membrane prior to cell entry and that interruption of lipid raft integrity significantly inhibited MV infection into HeLa cells (14). To gain more insight into the molecular mechanisms of MV entry, we aimed to identify cellular proteins that are enriched in lipid rafts upon vaccinia virus infection by quantitative proteomic analyses. Here, we show that one of the lipid raft-associated proteins, CD98, is important for vaccinia MV infection to host cells. Our results demonstrate that CD98 mediates MV endocytosis in both MEF and HeLa cells and that the entire structure of the CD98 protein is required to preserve its functions for mediating MV entry.

Materials and Methods

Cells and viruses. HeLa cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Sigma). MEF derived from CD98 conditional KO ES cells were described previously (48). MEFs were cultured in DMEM supplemented with 10% fetal bovine serum (HyClone), 0.1 mM nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 1% penicillin/streptomycin (Sigma), 1% HEPES (Invitrogen) and 0.2% 2-mercaptoethanol. The vaccinia virus Western Reserve (WR) strain was used in this study. The IHD-J strain of vaccinia virus and IA27L virus were obtained from Dr. G. L. Smith. The IA27L virus contains an A27L open reading frame (ORF) under the regulation of isopropyl-β-D-
thiogalactopyranoside (IPTG); therefore, it was grown in culture medium containing 5 mM IPTG (51). The truncated A26L ORF of IA27L virus was subsequently replaced by a full-length A26L (WR) ORF to generate a recombinant virus, IA27L-A26LWR, which was also grown in the presence of IPTG as described (12). The IHD-W strain of vaccinia virus was purchased from ATCC (ATCC 1441-VR). WR A4-mCherry virus was prepared as described (26). WR, IA27L-WRA26, IHD-J and IHD-W viruses expressing the dual expression cassette luc-lacZ (inserted at the tk locus) were subsequently generated with the luciferase (luc) gene driven by an viral early promoter and the lacZ gene by the p11k late promoter. WRA25L and WRA26L expressing a dual luc-gpt cassette containing the luciferase gene driven by a viral early promoter and the gpt gene by the p7.5k promoter were used as described (9).

Purification of vaccinia MV was performed as previously described (29).

Antibodies, reagents and plasmids. Mouse anti-human CD98 derived from the hybridoma cell line 4F2(C13) (anti-CD98) was purchased from ATCC (HB-22). The CD98 antibody was purified from the hybridoma culture medium by protein A affinity chromatography. Rabbit anti-CD98 polyclonal antibody (H300), goat anti-cyclophilin B polyclonal antibody (C15) and mouse anti-human CD69 monoclonal antibody (HP-4B3) were purchased from Santa Cruz Biotechnology. Mouse anti-β actin was purchased from Sigma (A5441). Mouse anti-GFP monoclonal antibody (JL8) was obtained from Clontech. Mouse anti-human CD69 antibody (F509), rat anti-integrin β1 antibody (9EG7, detects integrin β1 of mouse and human origin), rat anti-mouse CD98 antibody, and FITC-conjugated rat anti-mouse CD98 antibody (clone H202-141) were purchased from BD Pharmingen. Mouse monoclonal anti-HA antibody (16B12) was obtained from Covance. Mouse anti-human CD71 (MEM-75) and rabbit anti-Rab5 (GTX13253) antibodies were purchased from Genetex. Rabbit anti-GST antibody (G7781) was purchased from Sigma. Rabbit anti-A4 antiserum was raised against
recombinant viral A4 protein purified from bacteria. Rabbit anti-vaccinia virus antiserum was raised against MVs. The mouse monoclonal antibody (clone 2D5), recognizing the L1 protein, was obtained from Dr. Y. Ichihashi (27). Secondary antibodies (donkey or goat anti-rabbit, anti-rat, anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC), Texas Red, or cyanine 5 (Cy5) were purchased from Invitrogen Inc.. HRP-conjugated secondary antibodies (goat anti-mouse and anti-rabbit) were purchased from Jackson ImmunoResearch Inc.. HRP-conjugated bovine anti-goat antibody was purchased from Santa Cruz Biotechnology. Alexa Fluor-594-, Texas Red- or FITC-conjugated cholera toxin B subunit (CTB), FITC-conjugated transferrin, and Texas Red- or FITC-conjugated dextran (molecular weight of 10,000) were purchased from Invitrogen Inc..

The YFP-pReceiver-M15 vector was purchased from Genecopoeia Inc.. Plasmids encoding human CD98, C69T98E98, C98T98E69, C69T98E69 and CD69 were provided by Dr. Mark H. Ginsberg (University of California, San Diego). These constructs were cloned into the pReceiver-M15 vector to make the above chimera ORFs fused with the YFP ORF, respectively. The C98T69E98 chimera was constructed into the pReceiver-M15 vector using the following primers: 5'- GGCTGGGTACGCACCCGCGTTCCTGTCCTGTGTGCTGTAATG-3' (Forward) and 5'- GCGACAACGCAGCGCCTGGCCCCACTGATAAGGCAATGAGG-3' (Reverse) to PCR the CD69 transmembrane region; 5'- CGTTCGAACCAGGACCACTGATAAGGCAATGAGG-3' (Forward) and 5'- GCGACAACGCAGCGCCTGGCCCCACTGATAAGGCAATGAGG-3' (Reverse) to add the CD98 cytoplasmic domain, the 5'- CGTTCCAACCAGCAGGAGCCACCCGAAGGGAGT-3' (Forward) and 5'- CCCTCAGTCAGCCCGTGGGGAAGCGG-3' (Reverse) to add the CD98 extracellular domain. The pEGFP-C1 vector was purchased from Clontech Inc.. Eps15D95/295 (DN-Eps15) was kindly provided by Dr. Alice Dautry-Varsat (Pasteur...
Institute, Paris, France). Plasmids encoding wild-type dynamin 1 (WT-Dyn1), dynamin 1 with a K44A mutation (DN-dyn1 [Dyn1K44A]), WT-Dyn2, and DN-dyn2 (Dyn2K44A) were kindly provided by Dr. Sandra Schmid (Scripps Research Institute, CA). Plasmids encoding constitutively active (CA)(G12V)Cdc42, DN(T17)NCdc42, CA(G12V)Rac1 and DN(T17)NRac1 were obtained from the University of Missouri-Rolla cDNA Resource Center. GFP-DNcaveolin1 (Cav1D1-81) was constructed as described (26). Dynasore was a gift from Tomas Kirchhausen (Harvard Medical School and the CBR Institute for Biomedical Research, Inc., MA). Chlorpromazine hydrochlorid, amiloride and blebbistatin were purchased from Sigma Inc.. Jasplakinolide were purchased from Invitrogen Inc.. [14C] L-Leucine was purchased from PerkinElmer Inc..

Isolation of low-density detergent-insoluble membrane fractions on flotation gradients for differential IMID-H4/-D4 labeling and High-performance liquid chromatography/tandem MS (LC/MS/MS) analyses. Low-density detergent-insoluble membrane microdomains were isolated as described previously, with some modifications described below (14, 47). HeLa cells (10^8) were mock infected with serum-free medium or infected with VV in serum-free medium at an MOI of 20 PFU per cell for 30 minutes at 37°C, washed twice with ice-cold PBS to remove unbound virions, and lysed with 5.0 ml of ice-cold TNE buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA) containing 1% Triton X-100 (Merck), 1 mM NaF, and a cocktail of protease inhibitors (Roche). The cells were further incubated at 4°C for 30 min with gentle agitation, and then the cell lysates were centrifuged for 10 min at 3,000 rpm at 4°C in an Eppendorf 5415C centrifuge to remove nuclei and insoluble materials. The precleared supernatants were mixed with equal volumes of 80% (w/v) sucrose in TNE buffer and then placed in the bottoms of ultracentrifuge tubes. A discontinuous sucrose gradient was formed by overlaying the homogenates sequentially with
3.9 ml of 30% and 3.5 ml of 5% sucrose (w/v) in TNE buffer. These mixtures were centrifuged for 18 h at 166,000 x g (38,000 rpm) at 4°C in an SW41 rotor (Beckman). After centrifugation, the Triton X-100-insoluble, low-density material containing lipid rafts was visible as an opaque band migrating at the boundary between the 5 and 30% sucrose solutions. The gradient fractions were collected from the top, and a total of 11 fractions (1.0 ml/fraction) were collected and stored at –80°C. For detection of the ganglioside GM1, dot blot analyses were performed as described previously (3, 14). The resulting GM1-positive fractions were mixed, diluted four volume of cold TNE, and centrifuged an additional 2 h at 166,000 x g at 4°C to pellet the detergent-resistant material.

The pellets from the second centrifugation were solubilized in a 90 µl of formic acid (Fluka, 98%). 200 mg of solubilized pellets were incubated with 10 µl CNBr (Fluka) at 25°C overnight. Then formic acid was neutralized by ammonia bicarbonate (ABC, Sigma). Peptides were reduced by 20 mM DTT (Pharmacia) in 6 M urea (Sigma) and 2 M thiourea (Riedel-de Haen) for 1hr at 37°C and alkylated by iodoacetamide (IAA, Sigma) 1hr at 37°C. Seven volumes of 50 mM ABC were added followed by 4 mg trypsin (Promega) digestion (1:50) at 37°C overnight. The reduced tryptic peptides were desalted by C₁₈ reverse-phase traps (Opti-Lynx, optimize Technologies, Inc.), dried, and resolubilized in 200 ml of 2.535 M NH₃ (Aldrich) in 50% methanol. The pH was adjusted to 11 with 2.535 M NH₃. The lysine residues in mock and infected tryptic peptides were labeled with 12.5 mg of 2-methylthio-2-imidazoline (IMID-H₄, Aldrich cat. no. 15844-4) and deuterium-labeled IMID-D₄ 40°C overnight, respectively as previously described (45). Reaction was terminated by addition 10 ml of 10% trifluoroacetic acid (TFA, Riedel-de Haen). 100 mg of labeled peptides from light and heavy fraction were mixed and desalted again. The labeled peptides were fractionated by strong cation-exchange chromatography (SCX, 1mmx15cm). The peptide mixture from 200 µg of detergent-resistant material was fractionated by two-dimensional chromatography SCX-
reverse-phase liquid chromatography. The first dimension, SCX, was eluted with a linear
gradient of 0 to 300 mM KCl (Riedel-de Haen) in 5 mM ammonium formate (Aldrich), pH
3.0. The peptides eluted from the SCX column were trapped in second dimension reverse
phase with two C18 reverse-phase traps operating alternately; the bound peptides were
desalted and eluted at 2-min intervals and collected on a fraction collector; a total of 23
fractions were collected. The amount of peptide in each fraction was estimated from the UV
absorption at 214 nm.

Mass spectrometric analysis was performed on a nanoscale LC-tandem mass spectrometry
(quadrupole time-of-flight mass spectrometer; QStar XL; Applied Biosystems). The
instrument setup was as follows. The flow (150 µl/min) from the binary pump (Agilent 1100,
with solvent A (100% deionized water) and solvent B (90% acetonitrile) (J. T. Baker), both
solvents containing 0.1% formic acid) was split with two T-shaped connectors connected to a
self-packed precolumn (C18; 2 cm long; 150 µm internal diameter) with an appropriate flow
restrictor to give a column flow rate of 10 µl/min for sample loading and 200 to 300 nl/min
for sample elution from the analytical column. The sample of 2 to 5 µg was injected onto the
precolumn (15 mm long; 150-mm internal diameter; C18) via a 20-µl sample loop. The
analytical column (C18; 15 cm long; 75-mm internal diameter) was connected to a 15-mm
electrospray emitter (10-mm tip opening) by a 1-cm Teflon sleeve. The chromatographic
separation was performed with a 120-min gradient profile as follows: 2% B (0 to 4.5 min),
linear gradient of 2 to 10% B (4.5 to 5 min), 10 to 40% B (5 to 80 min), 40 to 50% B (80 to
100 min), 50 to 80% B (100 to 105 min), 80 to 2% B (105 to 106 min), and 2% B (106 to 120
min). The mass spectra of the eluted peptides were acquired in data-dependent mode by first
acquiring a full MS scan from m/z 400 to 1900 for 1 second to determine the three most
intense peptide peaks with charge states above 2, and then three MS/MS scans
between m/z 100 and 2000 (1.5 s each) were performed for the MS-scanned parent ions with a
threshold above 20 counts. Once sampled, each MS/MS precursor mass was excluded from further tandem experiments for 2 min. The data files completed from the LC-MS runs were converted to Mascot generic-format files using the Mascot.dll script supplied with the Analyst QS 1.1. The Mascot software package (Matrix Science) was used for database searching and protein identification using the Swiss-Prot Human database. Peptide mass tolerance and fragment mass tolerance were set at 100 ppm and 0.25 Da, respectively, for the initial search. An alternative calibration algorithm based on Mascot protein identifications was applied to the raw data file to give mass accuracies within 20 ppm. Ion score cut-off was 25.

Quantitation methods were performed by manual and MSquant.

**Immunofluorescence microscopy. (i). Patching analyses.** Patching of cell-surface proteins was performed as previously described (26, 52). HeLa cells were seeded on coverslips in 12-well plates and infected on the next day with vaccinia MV at a multiplicity of infection (MOI) of 50 PFU/cell in DMEM for 1 h at 4°C. Cells were washed with PBS, incubated at 37°C for different times and subsequently incubated for 1 h at 12°C with primary antibodies. Rabbit anti-vaccinia virus (1:500), mouse anti-human CD98 (1:2000), mouse anti human CD71 (1:250) and Alexa Fluor 594-cholera toxin B (CTB, 10 µg/ml) (Molecular Probes, Invitrogen) were used. Cells were washed at 4°C and incubated for 1 h at 12°C with secondary antibodies, Cy5-conjugated goat anti-rabbit antibody and FITC-conjugated goat anti-mouse antibody, respectively. DNA was visualized by staining with 0.5 µg/ml of DAPI (4',6-diamidino-2-phenylindole dihydrochloride; Molecular Probes). Cells were washed with PBS at 4°C and fixed for 30 min on ice with 3.7% paraformaldehyde in PBS. Images were collected with an LSM510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) using a 63x objective and confocal microscopy software (Zen 2009, Carl Zeiss). Co-localization analysis was performed using the confocal microscopy software (Zen 2009, Carl Zeiss). The Z-
sections of about 50 cells were analyzed and the percentage of virion-positive pixels showing co-localization with CD98-positive pixels was quantified. (ii) **Virion binding and uncoating assays.** HeLa cells and MEFs were seeded on coverslips for 24 h and 48 h, respectively, before the experiment. The virion binding assay measuring the amount of cell surface-bound virions was performed as described previously (56). The virion penetration assay measuring the intracellular uncoated cores, which can be stained only with anti-A4 core Ab after virus entry, was also performed as described previously (55). Cells were infected for 1 h at 4°C with vaccinia MV at an MOI of 20 PFU/cell for the binding assay and 40 PFU/cell for the uncoating assay, washed three times with PBS, and either fixed immediately (for the virion binding assay) or incubated for 2 h at 37°C in the presence of cycloheximide (30 µg/ml) and then fixed (for the virus penetration assay). The cells were permeabilized in PBS containing 0.2% saponin, and stained with rabbit anti-A4L antibody or mouse anti-L1R monoclonal antibody, followed by Texas Red-conjugated goat anti-rabbit antibody or FITC-conjugated goat anti-mouse antibody, respectively. DNA was visualized by staining with 0.5 µg/ml of DAPI. Images were collected with an LSM510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) using a 63x objective and confocal microscopy software (Zen 2009, Carl Zeiss). Fluorescent particles from multiple images were counted, and the average number of surface-bound virions and uncoated cores per cell were determined as described previously (26). (iii) **Ligand and antibody internalization assays.** HeLa and MEF cells were seeded on coverslips for 24 h and 48 h, respectively, before the experiment. For antibody internalization assays, cells were incubated for 1 h at 4°C in PBS-AM (PBS, 0.05% bovine serum albumin, 10 mM MgCl2) in the presence of 2.5 µg/ml FITC-conjugated rat anti-mouse CD98 antibody and WR-mCherry MV at an MOI of 20 PFU/cell. Cells were then shifted to 37°C to allow endocytosis of the antibody and the virus. After internalization, surface antibody was removed by low-pH, acidic washing (0.2 M acetic acid, 0.5 M NaCl, pH 2.5) prior to fixation. Staining
of intracellular phosphatidylinositol-3-phosphate (PI3P) was performed based on an established protocol previously described (23). In brief, after the acid washes, the cells were further incubated for 10 min with 20 µg/ml GST (as a negative control) or GST-FYVE recombinant protein in 0.2 % saponin (Sigma) diluted in 1x PBS, washed and then fixed. Images were collected with an LSM510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) using a 63x objective and confocal microscopy software (Zen 2009, Carl Zeiss).

For transferrin, dextran and CTB uptake analyses, cells were starved in serum-free DMEM for 1 h at 37°C and then incubated for 30 min at 37°C in complete medium containing 25 µg/ml Texas Red-conjugated transferrin (Invitrogen Inc.) as previously described (26). For CTB and dextran uptake, cells were incubated for 40 min at 37°C in complete medium containing 5 µg/ml of Alexa Fluor 594-conjugated CTB or 5 mg/ml of FITC-conjugated dextran (MW 10,000; Invitrogen). Subsequently, cells were washed extensively to remove extracellular-bound ligands and fixed as previously described (26).

**Inhibitor experiments and virus entry assays.** For inhibitor experiments, cells were pretreated with each of the inhibitors prior to virus infections as described below. For bafilomycin A (BFLA) blocking experiments, cells were pre-treated in complete DMEM containing DMSO or BFLA (0.5, 1, 5, 10, 25 or 50 nM) for 30 min. For dynasore, chlorpromazine (Cpz) and amiloride blocking experiments, cells were pre-incubated with DMSO, dynasore (40 µM or 80 µM), Cpz (2.5 µg/ml or 5 µg/ml) or amiloride (0.5 mM, 1 mM, 2.5 mM or 5 mM) in serum-free DMEM for 1 h (for dynasore, 30 min incubation) at 37°C. For jasplakinolide and blebbistatin blocking experiments, cells were pre-treated in complete DMEM containing 250 nM jasplakinolide, blebbistatin (10 µM or 25 µM), or 250 nM jasplakinolide and blebbistatin (10 µM or 25 µM). These drug-treated cells were subsequently infected with vaccinia MV at an MOI of 5 PFU/cell, for early gene luciferase...
expression assays (54), or cooled to 4°C and infected at an MOI of 40 PFU/cell for 1 h at 4°C for virus core uncoating assays (55). After washing with PBS, cells were incubated in growth medium for 2 h at 37°C and harvested for luciferase assays (54). Alternatively, cells were incubated in growth medium for 2 h at 37°C in the presence of cycloheximide (30 µg/ml) and then fixed for virus uncoating assays (55). The drugs were present in the medium throughout the experiments. For cellular ligand uptake experiments, cells pretreated with dynasore and Cpz as described above were subsequently pulsed with fluorescent-conjugated transferrin for 30 min, washed with PBS, and then fixed as previously described (26).

**siRNA knockdown.** The control cyclophilin B (CypB) small interfering RNA (siRNA) duplex (Accell SMARTpool PPIB) and the two CD98 siRNA duplexes (Accell SMARTPool SLC3A2) were purchased from Dharmacon Inc.. HeLa cells were transfected with siRNA duplexes (50 pM) using Lipofectamine 2000 (Invitrogen), the process was repeated, and then the cells were used for virus binding and penetration assays as previously described (26, 55, 56).

**Flow cytometry.** Immunolabeled cells were analyzed on a flow cytometer (FACSCalibur, BD) using the CellQuest (BD) software. CD98 expression levels on MEFs were measured by rat anti-mouse CD98 (BD Pharmingen) and the mouse anti-human CD98 antibodies. Mouse and human integrin β1 was measured with a rat anti-integrinβ1 antibody (9EG7, BD Pharmingen).

**Cell fusion assays (fusion-from-without).** Cell fusion assays were performed as described previously (9). MEFs were seeded in 96-well plates for 48 h, pre-treated with 40 µg/ml cordycepin (Sigma) for 60 min and subsequently infected with WR, WRAA26L, IA27L, or
IHD-W MVs at an MOI of 100 PFU/cell in triplicate. After infection for 60 min at 37°C, cells were incubated in complete DMEM supplemented with 40 µg/ml cordycepin and incubated for 2 h at 37°C. Cells were fixed and stained with DAPI (0.5 µg/ml) and the fluorescent lipid dye PKH26 (Sigma) (11). Cell images were collected with an LSM510 Meta confocal scanning microscope (Carl Zeiss) using a 40x objective and confocal microscopy software (Zen 2009, Carl Zeiss).

**Amino acid uptake assays.** The assays were performed as previously described (32). In brief, cells were washed three times with a standard uptake solution (125 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES, 1.2 mM KH₂PO₄ and 5.6 mM glucose, pH 7.4) and incubated for 1 min in uptake solution containing 1 µCi/ml [14C] L-leucine. After washing three times with ice-cold uptake solution, the cells were solubilized with 0.1 N NaOH, and the radioactivity was measured using a liquid scintillation counter.

**Results**

**CD98 is enriched in lipid rafts upon vaccinia MV infection**

Because plasma membrane lipid rafts are required for vaccinia virus entry (14), we investigated whether any specific host factors or receptors are enriched in lipid rafts for virus binding and/or penetration. A lysine-residue specific isotope labelling scheme was utilized to determine the variation of relative abundance before and after vaccinia virus infection. We therefore isolated low-density, detergent-resistant membrane microdomains (DRM) before and after vaccinia virus infection of HeLa cells as previously described (3, 14, 47). Proteins were extracted from DRM, digested to peptides, differentially labeled with 2-methylthio-2-imidazoline (IMID-light (D0)) and deuterium-labeled IMID ((IMID-heavy (D4)),

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respectively, with the established methods described previously (45), and analyzed by multi-dimensional high-performance liquid chromatography/tandem MS (LC/MS/MS) (Figure 1A). A total of 717 cellular proteins were identified and among those, 570 proteins (79%) were quantified (Supplemental S1A). The relative abundance of these quantified proteins fluctuated between 0.57 and 1.97-fold, with an average of 1.06±0.11 (Supplemental S1B), before and after virus infection, suggesting that the levels of the majority of DRM-associated proteins were not drastically changed after virus infection. In fact, 97% of all quantified proteins fell within the range of the ratio of 1.0±0.5, i.e., a ratio that was considered not significantly changed. Interestingly, among these DRM-associated proteins, the level of CD98/SLC3A2 was increased 1.75-fold after vaccinia virus infection (Table1).

Following the proteomic analyses, we performed co-patching of lipid raft experiments by staining HeLa cell-surface CD98 and found co-localization of CD98 with the lipid rafts marker cholera toxin B (CTB) (Figure 1B), confirming CD98 as a DRM-associated membrane protein. Furthermore, when HeLa cells were infected with vaccinia virus Western Reserve (WR) at 37°C for 5 min, co-localization of vaccinia MV with CD98 and CTB was detected (Figure 1C). As a control, we performed co-patching analysis using another receptor, the transferrin receptor (CD71) (Figure 1D). After quantifying WR MV co-localization with CD98 or CD71, we confirmed that a significant amount of MV co-localized with CD98 as compared to CD71 (Figure 1E). Altogether, these results imply a role of CD98 in vaccinia virus infection.

**CD98 depletion in HeLa cells reduces vaccinia WR MV endocytosis**

To investigate whether CD98 is important for MV entry, we knocked down its expression in HeLa cells using siRNA (si-CD98). HeLa cells either mock-treated or treated with siRNA knocking down cyclophilinB expression (si-CybB) were used as controls (Figure...
We then tested these knockdown cells for infectivity of two MV particles, the wild-type WR strain and a recombinant WR strain, IA27L (12, 51), because the former enters HeLa cells via endocytosis whereas the latter fuses with the plasma membrane (9). HeLa cells were infected at an MOI of 5 PFU/cell and harvested at 2 h post-infection (p.i.) for early gene luciferase expression assays (Figure 2B). The results suggested that depletion of CD98, but not CypB, reduced vaccinia WR infections to 50% whereas it had no effect on IA27L infectivity. To investigate whether CD98 is important for WR virus entry step, we performed MV binding assays, which determine the amount of cell-bound MV particles prior to membrane fusion (56), and virus core uncoating assays, which measures the number of viral cores after virus membrane fusion (55). As shown in Figure 2C, knockdown of CD98 had no effect on WR or IA27L MV binding to HeLa cells (Figure 2C), demonstrating that CD98 is not involved in the attachment of either virus to HeLa cells. On the other hand, CD98 knockdown significantly reduced the intracellular viral core number of WR virus but not IA27L virus (Figure 2D, and quantified in 2E). Taken together, our results suggest that CD98 is specifically required for the endocytic route of vaccinia MV into HeLa cells.

Vaccinia WR enters MEF via a CD98-dependent fluid phase endocytic pathway while IA27L virus enters MEF via CD98-independent plasma membrane fusion

In order to confirm our above observations in HeLa cells, we next obtained MEF derived from CD98 conditional knockout (KO) mice embryos that either expressed an endogenous level of CD98 (CD98+/+) or no CD98 (CD98/-) (48). Before we addressed the role of CD98 in vaccinia virus entry we first characterized vaccinia WR and IA27L MV entry pathways in MEF to ensure there is no cell type-specific difference between HeLa and MEF. Bafilomycin treatment of MEFs at a range of 5-50nM inhibited WR MV entry but not IA27L virus (Figure 3A) suggesting that WR MV, but not IA27L, enters MEF cells through a low-
pH dependent endocytosis. On the contrary, IA27L MV, but not WR, entered MEFs through plasma membrane fusion resulting in robust cell fusion-from-without at neutral pH (Figure 3B). To further dissect the WR MV endocytic pathway in MEF cells we used different inhibitors and dominant negative (DN) constructs that specifically block individual endocytic pathways. The results revealed that, similar to HeLa cells, vaccinia WR MV is endocytosed into MEFs through a clathrin-independent, dynamin-dependent (Figure 4A) and caveolin-independent (Figure 4B) endocytic pathway. Control experiments showed that transferrin uptake into MEF was reduced by inhibitors such as chloropromazine (Cpz), dynasore, DN-dynamin 1 and 2 and DN-Eps15, all of which inhibited clathrin-mediated endocytosis (Figure 4C) and that cholera toxin B (CTB) uptake through caveolae was blocked by DN-cav1 (Figure 4D). Finally, vaccinia WR MV endocytosis into MEF was sensitive to the inhibitors of actin dynamics (Figure 5A), GDP-bound Rac and CDC42 mutants (Figure 5B) and the macropinocytosis inhibitor amiloride (Figure 5C). Overall, the results demonstrated that, vaccinia WR MV enters MEFs in a dynamin-dependent fluid phase endocytic manner whereas IA27L MV entry was through plasma membrane fusion (Results are summarized in Table 2).

We next compared MV entry into wild type (CD98+/+) and CD98 KO (CD98-/-) MEF cells. In addition, plasmid constructs expressing yellow fluorescent protein (YFP) alone or YFP fused with CD98 were individually transfected into CD98-/- MEF to generate two reconstituted cell lines stably expressing either YFP (CD98-/- +YFP) or YFP-CD98 protein (CD98-/- +YFP-CD98) (Figure 6A). We then performed infections with vaccinia WR and IA27L MV on all four MEF cell lines as described above and harvested cells to monitor virus entry. Vaccinia WR and IA27L MV binding to all four MEF cell lines were comparable (Figure 6B), whereas viral core uncoating (Figure 6C) and subsequent early luciferase gene expression (Figure 6D) of WR MV, but not IA27L, was reduced by 60% in CD98-/- MEF.
Reconstituted expression of YFP-CD98, but not YFP, in CD98-/- MEF restored WR MV infectivity (Figure 6C and D). Imaging analyses consistently showed a reduction in the number of viral cores in CD98-/- MEF cells infected with WR (Figure 6E) and a specific rescue of WR infectivity by overexpression of YFP-CD98 (Figure 6F). Taken together, our results showed that CD98 is important for vaccinia WR MV entry into MEFs but not for IA27L virus.

Different strains of vaccinia MV require CD98 during endocytic entry

Since vaccinia virus entry was shown to be strain-specific we wanted to corroborate this conclusion by including several vaccinia virus strains and deletion mutants whose entry pathways into HeLa cells have been well characterized (9). For example, wild-type WR, the IHD-J strain and IA27L-A26WR, a recombinant IA27L expressing full-length A26 protein, use the endocytic entry route, whereas the IA27L strain, IHD-W and a deletion mutant virus, WRA26L, enter HeLa cells through plasma membrane fusion (9). We thus infected CD98+/+ and CD98-/- MEF with each MV at an MOI of 5 PFU/cell and performed early gene luciferase expression assays at 2 h p.i.. As shown in Figure 7A, all the endocytic MVs (WR, IHD-J, and IA27L-A26WR) had a reduced infectivity in CD98-/- MEFs when compared to CD98+/+ MEFs. Furthermore, the reduced infectivity of the above viruses, such as WR and IA27L-WRA26, in CD98-/- MEFs was not due to a delay kinetics in MV entry as longer exposure of viruses with the target cells did not increase virus infectivity in CD98-/- cells (Figure 7B). In contrast, the MVs targeting the plasma membrane fusion route, such as IA27L and WRA26L, showed comparable infectivity on both CD98+/+ and CD98-/- cells (Figure 7C). Consistently, these latter viruses triggered robust cell fusion-from-without at neutral pH on both CD98+/+ and CD98-/- MEFs (Figure 7D), demonstrating that CD98 is dispensable for the vaccinia virus-induced plasma membrane fusion. These results show that
vaccinia MV strains, which enter cells through endocytosis, not plasma membrane fusion, require CD98 for cell entry.

Vaccinia WR MV localizes in endocytic structures in cells that are positive for CD98

If CD98 is required for vaccinia virus endocytosis, it is anticipated that vaccinia MV is internalized into CD98-positive intracellular vesicles. One approach to investigate this issue is to use anti-CD98 mAb to track the internalization of CD98 from the plasma membrane into intracellular vesicles (19). This method has been widely used and shown not to alter the ligand binding and intracellular trafficking of the targeted plasma membrane proteins (19). Accordingly, wild-type MEFs were incubated with an FITC-conjugated anti-CD98 mAb together with a fluorescent vaccinia WR MV expressing the viral core protein A4 fused with mCherry for 1 h at 4°C to allow the virus and the antibody to bind the cells. These cells were then incubated at 37°C for 5, 15, 30 or 45 min to allow the occurrence of internalization, washed extensively with acidic buffer to remove any extracellular-bound antibody, and fixed for confocal microscopy analyses. Individual Z-sections of images of cells at the different time points are shown in Figure 8. At time $t=0$ min, all surface-bound FITC-conjugated anti-CD98 mAbs were not internalized and thus effectively removed by washing prior to fixation (Figure 8A). Cells fixed at 5 min and 15 min contained detectable acid-resistant FITC fluorescence, mostly at the cell periphery, suggesting that initial internalization of surface CD98 occurred (Figure 8B and C). Furthermore, vaccinia MVs were found within internalized CD98-positive vesicles as soon as 5 min after the temperature shift to 37°C (arrowheads). Images of cells at 15, 30 and 45 min after the temperature shift showed that vaccinia MV was located within CD98-positive vesicles that already trafficked away from the cell periphery (Figure 8D and E). The majority of infected cells ($\geq 80\%$) contained MV particles located within CD98-positive vesicles as early as 5 min after temperature shift to 37°C (Figure 8F).
Furthermore, 40-50% of internalized MV particles in cells localized inside CD98-positive vesicles at 5 and 15 min (Figure 8G) demonstrating that CD98 and vaccinia WR MV co-
internalization occurred very quickly after the temperature shift.

To characterize the identity of the above-shown CD98-positive vesicles that contain WR-MV, we performed the antibody-internalization assays in the presence of WR MV and immunostained for various cellular endocytic markers after fixation. We observed co-localization of CD98-positive vesicles containing WR MV with phosphoinositol-3-phosphate (PI3P), a marker for macropinosomes (31) (Figure 9A, arrowheads), and the early endosome markers Rab5 (Figure 9B, arrowheads). These results show that WR MV and CD98 are internalized into PI3P-positive macropinosomes, which subsequently fused with early endosomes.

Expression of CD98-CD69 chimeras in CD98-/- MEF reveals that the full-length CD98 is required to facilitate vaccinia WR MV endocytosis

The above results prompted us to investigate whether the cellular function of CD98 is important for vaccinia MV endocytosis. CD98 is known to associate with light chains of amino acid (aa) transporters and modulates amino acid transport into cells (5, 53). CD98 also associates with integrin β1 and regulates integrin downstream Akt signaling (20, 22). It was previously shown that the aa transporter activity of CD98 depends on its extracellular domain whereas the integrin signaling activity requires transmembrane and cytoplasmic domains of CD98 (21).

A series of four chimeric constructs were described before (21) in which domains of CD98 were replaced with portions of another type II transmembrane protein, CD69. These four chimeric CD69-CD98 constructs (Figure 10A) were fused with YFP, transfected individually into CD98-/- MEF, and the resulting stable cell lines expressing each of the chimeric YFP-
fusion proteins at comparable levels were confirmed by both FACS (Figure 10B) analysis and immunoblot (Figure 10C). These cells were subsequently infected with vaccinia WR MV and harvested at 2 h p.i. for early gene luciferase assays (Figure 10D) and virus core uncoating assays (Figure 10E) as described above. The results showed that while ectopic expression of full-length YFP-CD98 reconstituted WR MV infectivity to about 80%, most of the CD69-CD98 chimeric proteins did not significantly increase virus infectivity when compared to control YFP or YFP-CD69 (Figure 10D and E). We observed only a moderate increase with three chimeras (C69T98E98, C98T69E98, and C98T98E69), which did not share a specific domain structure. Thus, the results suggest that the entire CD98 molecule is required to facilitate WR MV endocytosis.

To analyze the functions associated with the CD98 domains in the chimeric proteins used, we examined which of the constructs could reconstitute amino acid transport in CD98-/- MEFs by performing [14C] L-leucine uptake assays as previously described (32) and quantifying the intracellular radioactivity with a liquid scintillation counter. [14C] L-Leucine uptake was very low in CD98-/- MEFs as well as in CD98-/- MEFs expressing YFP or YFP fused with CD69 or a construct containing the extracellular and cytoplasmic domains of CD69 (C69T98E69) (Figure 10F). The chimeras containing the extracellular domain of CD98 (C69T98E98, C98T69E98), and the construct comprising the cytoplasmic and transmembrane domain of CD98 but lacking its extracellular domain (C98T98E69) partially reconstituted aa transport activity (Figure 10F). These results demonstrate that chimeras lacking one of the three domains (i.e., the extracellular, the transmembrane or the cytoplasmic domain) of CD98 are still able to transport the light chain to the plasma membrane and induce amino acid transport, albeit at a reduced efficiency. To reconstitute complete amino acid transport activity, however, all three domains of CD98 were required. Taken together, our results show that aa transport activity appears necessary but not sufficient to explain the fact that the entire
CD98 protein is required for MV endocytosis, suggesting that additional functions provided by full-length CD98 are important for MV entry.

Discussion

The identification of cellular receptors employed by vaccinia virus to enter different types of cells represents an important step of virus research, and a detailed understanding of the cellular targets of vaccinia virus will help to learn more about the virus tropism and pathogenesis. Although several receptors were reported to participate in vaccinia virus entry, evidence pertaining to their significance is still lacking (18, 34, 37, 50). Here, we show that CD98 is enriched in the host cell membrane upon vaccinia MV infection and that CD98 and vaccinia MV co-localize in lipid rafts. Moreover, we demonstrate that WR MV can be found within CD98-positive endocytic vesicles. In addition, by using CD98 KO MEFs and YFP-CD98 reconstituted MEFs as well as CD98 knockdown in HeLa cells, we here demonstrate that the type II transmembrane glycoprotein CD98 plays a role in vaccinia WR strain MV entry through endocytosis.

Interestingly, CD98 is widely expressed on multiple cell types (49, 60), which correlates with the broad spectrum of vaccinia virus infectivity. This could explain why vaccinia MV targets this cellular receptor in order to gain entry into many different cell types. CD98 was previously shown to be endocytosed in HeLa cells in a clathrin- and dynamin-independent manner (19). Our study shows vaccinia WR strain is endocytosed into MEF cells in a non-clathrin-, non-caveolae-, but dynamin-dependent pathway which is similar to what was previously observed in HeLa cells (26). Our findings are in agreement with the previously described CD98-endocytic route, with the exception that vaccina WR MV endocytosis required dynamin GTPase activity, while CD98 endocytosis itself does not (19).
This suggests that vaccinia MV targets CD98 first in order to initiate the fluid-phase endocytic uptake, and then subsequently recruits dynamin to accommodate the membrane fusion event.

Although CD98 was initially identified as a molecule involved in cell-cell fusion (28, 41-44), it is not required for vaccinia MV membrane fusion per se. In our fusion-from-without assays, the CD98 KO MEFs underwent cell-cell fusion more rapidly than the CD98+/+ cells, suggesting that CD98 plays a minor role in the regulation of the vaccinia MV-induced cell-cell fusion. Nevertheless, our study shows clearly that the whole structure of CD98 is essential for vaccinia WR MV endocytosis.

CD98 accomplishes two main cellular functions: by forming heterodimers with several light chains, it functions as an amino acid transporter (HATs) (10, 59), and it interacts with integrins to modulate integrin-dependent signaling (20, 22, 63). We performed RT-PCR analysis and found that several CD98-associated light chains, i.e., LAT1, LAT2, y+LAT1, y+LAT2, and xCT were expressed in HeLa and MEF cells (data not shown). While previous studies showed that the extracellular domain of CD98 is required for its association with its light chain and for mediating amino acid transport (21), others have demonstrated that elimination of the disulfide bridge that forms between CD98 and the light chain or the extracellular domain of CD98 does not completely abolish the expression of the heterodimer at the plasma membrane and the transport of amino acids (6, 40, 46, 61). Using CD98-CD69 chimeric constructs, we found that even though some chimeras could partially reconstitute amino acid transport, none of them reconstituted WR infectivity in MEF cells. Thus, we concluded that activation of amino acid transport by CD98 is necessary but not sufficient to explain the role of CD98 in WR infectivity. One reason could be that the CD98-CD69 chimeric constructs are not efficiently endocytosed or are not endocytosed at the same rate as wild-type CD98 does. Another function of CD98 is to regulate raft-associated receptor functions such as integrin β1 (20, 22, 63). In that aspect, another study recently conducted in
our laboratory revealed that integrin $\beta_1$ plays an important role in the binding and entry of WR MV into HeLa cells (Izmailyan et al., submitted).

Previously, other viruses were shown to target CD98 or amino acid transporters. The cationic amino acid transporter CAT1 was shown to be required for moloney leukemia virus (MLV) to enter rodent cells (1, 33). The CD98 light chain xCT is also suggested to play a role in the entry (30) and replication (57) of Kaposi’s sarcoma-associated herpesvirus (KHSV).

In summary, our study demonstrates that CD98 is specifically required for vaccinia WR MV endocytic entry into HeLa cells and MEFs. No single CD98 domain is sufficient to provide help for vaccinia MV entry, suggesting that vaccinia MV requires both functions of CD98 i.e., the regulation of amino acid transport as well as integrin $\beta_1$ signaling. Our results, however, do not exclude the possibility that vaccinia virus simply targets the overall structure of CD98, irrespective of its functions in cells, to gain entry into the cells. Whether such structural requirement of CD98 is influenced by its associated molecules such as integrin $\beta_1$ and light chains of amino acid transporters is currently unknown. Further studies are needed to better understand how CD98 participates in vaccinia virus endocytosis.

Acknowledgements

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**Figure legends**

**Figure 1. Vaccinia MV particles co-localize with cell surface CD98 in lipid rafts upon vaccinia MV infection.** (A) Flow chart of differential IMID-H4/-D4 labeling and LC/MS/MS analyses of lipid raft-associated proteins isolated from HeLa cells that were either mock-infected or infected with vaccinia WR strain MV. Experimental details are described in materials and methods and the list of identified and quantified raft-associated cellular proteins is included in Supplemental S1. (B) CD98 localizes at the plasma membrane lipid rafts. HeLa cells were co-patched with mouse anti-human CD98 antibody (green) and the lipid raft marker cholera toxin B (CTB-Alexa Fluor 594, red) without permeabilization as described in
materials and methods. (C and D) Vaccinia WR MV copatched with CD98 on cell surface lipid rafts. WR MV was allowed to bind to HeLa cells for 1h at 4°C, washed and the cells were transferred to 37°C for 5 min before co-patching with (C) mouse anti-human CD98 (green) and rabbit anti-vaccinia virus (cyan) antibodies and the lipid raft marker cholera toxin B (CTB, red) or with (D) mouse anti-human CD98 (red) or mous anti-human CD71 (red) and rabbit anti-vaccinia virus (green) antibodies without permeabilization. (E) Quantification of vaccinia WR MV particles co-localization with CD98 shown in D. The data were quantified by calculating the fraction of total of WR MV-positive pixels co-localizing with CD98- or CD71-positive pixels per cell. A total of ~ 50 cells were quantified and the statistical analyses were performed using Student’s t-test. The P-value is shown ***, P < 0.0001.

Figure 2. CD98 depletion in HeLa cells reduces vaccinia WR MV endocytosis. (A) Immunoblots of HeLa cell lysates prepared from mock-treated cells (Mock) or cells transfected with CypB siRNA (si-CypB) or CD98 siRNA (si-CD98) with antibodies against CD98, cyclophilin B, or β-actin (control). (B) Early gene luciferase expression assays in HeLa cells. Mock-, si-CypB and si-CD98 treated HeLa cells were infected with WR or IA27L MV at an MOI of 5 PFU/cell for 60 min and harvested at 2 h p.i. for early gene luciferase expression assays. Values for mock-treated HeLa cells were set as 100%. (C) Quantitative results of virion binding assays. Mock-, si-CypB and si-CD98 treated HeLa cells cells were infected for 60 min at 4°C with WR or IA27L MV at an MOI of 20 PFU/cell, washed and subsequently fixed with 4% paraformaldehyde. The bound particles were stained using an anti-L1R mAb 2D5. Bound virions of 40 cells were counted in each experiment to obtain an average number of bound virions/cell. The number of bound virions obtained from mock-siRNA cells (control) was set as 100%. The experiment was performed three times and the standard deviation is shown. (D) Immunofluorescence microscopy of virus uncoating assays
in Mock-, si-CypB and si-CD98 treated HeLa cells infected with WR or IA27L MV. The core was stained using the anti-A4 antibody (red) and the nuclei were stained with DAPI (blue).

(E) Quantitative results of the virus uncoating assays shown in D. Viral core numbers in 40 cells were counted to obtain an average number of viral cores/cell. Viral core number obtained from mock-treated HeLa cells was used as 100% control. The experiments were performed three times and the standard deviations are shown.

Figure 3. Vaccinia WR MV enters CD98+/+ MEFs through endocytosis. (A) Bafilomycin A (BFLA) sensitivity of WR and IA27L MVs in MEFs. CD98+/+ MEFs were pre-treated with DMSO or BFLA for 30 min and subsequently infected with WR or IA27L MV at an MOI of 5 PFU/cell for 60 min at 4°C. Cells were harvested and assayed for early luciferase gene expression 2 h p.i.. Luciferase activities obtained from MEFs treated with DMSO were used as 100% control. (B) IA27L but not WR triggers fusion-from-without at neutral pH. CD98+/+ MEFs were infected with WR or IA27L at an MOI of 100 PFU/cell for 60 min at 37°C at neutral pH. At 2 h p.i., cells were fixed, stained with DAPI and a fluorescent lipid dye (PKH26).

Figure 4. Vaccinia WR MV entry in MEFs is independent of clathrin- and caveolin-mediated endocytosis but relies on dynamin GTPase activity. (A) Vaccinia MV uncoating assays in HeLa and MEF cells pretreated with inhibitors of clathrin-mediated endocytosis and dynamin GTPase activity. HeLa cells and MEFs were transfected with plasmids for the expression of GFP, dominant negative (DN)-Eps15, DN-Dyn1 and DN-Dyn2 or pre-treated with DMSO, chlorpromazine (Cpz) or dynasore and infected with WR at an MOI of 40 PFU/cell and virus core numbers in cells were determined as described in materials and methods. In each group, at least 40 cells were counted to obtain an average number of viral
cores/cell; the core numbers obtained from DMSO-treated cells (for drug treatments) and GFP-transfected cells (for transfections with DN constructs) were used as 100% control. (B). Vaccinia MV uncoating in HeLa and MEF cells transfected with DN-caveolin-1. HeLa and MEFs were transfected with plasmids expressing GFP or GFP-DN-caveolin1 (DN-Cav1) and infected with WR MV at an MOI of 40 PFU/cell and harvested for virus uncoating assays as described in A. (C) Transferrin uptake in HeLa and MEF cells pretreated with inhibitors or plasmid transfections as described in A. The treated cells were subsequently pulsed with fluorescent transferrin for 30 min, washed, fixed and analyzed by confocal microscopy to quantify the uptake of fluorescent transferrin ligand. (D) Cholera toxin B (CTB) uptake in transfected HeLa and MEF cells as described in B. The transfected cells were pulsed with fluorescent CTB for 40 min, washed, fixed and analyzed by confocal microscopy to quantify the uptake of fluorescent CTB ligand. All the experiments were repeated three times and the standard deviations are shown.

Figure 5. Vaccinia WR MV enters MEFs through macropinocytosis (A) Myosin inhibition and blockage of actin dynamics. HeLa cells and MEFs were pre-treated with DMSO, blebbistatin (B), jasplakinolide (J) or blebbistatin along with jasplakinolide (J+B), infected with WR MV at an MOI 5 PFU/cell for 60 min and harvested at 2h p.i. for for early gene luciferase expression assays. Luciferase activities obtained from the DMSO-treated cells were used as 100% control. (B) Small GTPase inhibition on vaccinia core uncoating. HeLa and MEFs cells were transfected with plasmids for the expression of GFP, GTP-bound form (G12V) and GDP-bound (T17N) forms of Rac1 and Cdc42. Cells were infected with WR MV at an MOI of 40 PFU/cell for virus core uncoating assays. In each group, at least 40 cells were counted to obtain an average number of viral cores/cell; viral core numbers obtained from GFP-transfected cells were used as 100% control. (C) Inhibition of macropinocytosis on
vaccinia core uncoating. HeLa cells and MEFs were treated with various concentrations of amiloride (Am) and subsequently infected with WR MV for virus core uncoating assays as described in B. Viral core numbers obtained from DMSO-treated cells were used as 100% control. All the experiments were repeated three times and the standard deviations are shown.

Figure 6. CD98 is important for vaccinia WR MV entry into mouse embryonic fibroblasts. (A) CD98 expression in CD98+/+ and CD98-/- MEFs. CD98+/+, CD98-/- and CD98-/- MEFs stably expressing YFP (CD98-/- YFP) or YFP-CD98 (CD98-/- YFP-CD98) were stained with rat anti-mouse CD98 (for CD98+/+ and CD98-/- cells) or mouse anti-human CD98 (for CD98-/- YFP and CD98-/- YFP-CD98 cells) antibodies and analyzed by FACS. (B) Vaccinia MV binding assays on MEF cells. The above-mentioned MEFs were infected with WR or IA27L MV at an MOI of 20 PFU/cell at 4°C for 60 min, washed and subsequently fixed with 4% paraformaldehyde. The bound particles were stained using an anti-L1R antibody (2D5). In each group, at least 40 cells were counted to obtain an average number of viral cores/cell; the number of bound virions obtained from CD98+/+ MEFs was used as 100% control. The experiments were performed three times and the standard deviations are shown. (C) Vaccinia virus uncoating assays on MEF cells. The above-mentioned were infected with WR or IA27L MV at an MOI of 40 PFU/cell and the viral core numbers were determined by staining with anti-A4 antibody. In each group, at least 40 cells were counted to obtain an average number of viral cores/cell; the viral core number obtained from CD98+/+ MEF cells was used as 100% control. The experiments were performed three times and the standard deviations are shown. (D) Early gene luciferase expression assays in MEF cells. MEF cells were infected with WR or IA27L at an MOI of 5 PFU/cell for 60 min and harvested at 2 h p.i. for early gene luciferase expression assays. Luciferase activities in CD98+/+ MEFs were used as 100% control. The experiments were performed three times and
the standard deviations are shown. (E) Immunofluorescence microscopy of virus uncoating
assays of CD98+/+ and CD98-/- MEF cells infected with WR or IA27L MV. The core was
stained using anti-A4 antibody (red). (F) Immunofluorescence microscopy of virus uncoating
assays of CD98-/- MEF cells expressing YFP-CD98 or YFP alone (green). MEF cells were
infected with WR MV at an MOI of 40 PFU/cell and viral cores were stained with anti-A4
antibody (red) as described in E.

Figure 7. Vaccinia MV strains that enter cells through endocytosis require CD98. (A)
Infectivity of WR, IHD-J, IA27L-A26WR, IA27L, IHD-W and WRΔA26L in CD98+/+ vs.
CD98-/- MEFs. Pairwise MEFs were infected with various viruses at an MOI of 5 PFU/cell
for 60 min and harvested at 2 h p.i. for early gene luciferase expression assays. Luciferase
activities obtained from the CD98+/+ MEFs were used as 100% control for each virus strain
tested. (B) Time course of WR, IA27L-A26WR, IA27L, and WRΔA26L infectivity in
CD98+/+ vs. CD98-/- MEFs. Pairwise MEFs were infected with various viruses at an MOI of
10 PFU/cell for 60 min and harvested at 30 min, 60 min, 90 min and 120 min p.i. for early
gene luciferase expression assays. Luciferase activities obtained from the CD98+/+ MEFs
were used as respective 100% control for each virus strain tested. (C) Time course of IA27L
and WRΔA26L infectivity in CD98+/+ vs. CD98-/- MEFs. Pairwise MEFs were infected with
various viruses and harvested for early gene luciferase expression assays as described in B.
(D) Fluorescence microscopy of cell fusion-from-without on CD98+/+ and CD98-/- MEFs
triggered by IA27L, IHD-W and WRΔA26L at neutral pH. Pairwise MEFs were infected with
IA27L, IHD-W or WRΔA26L at an MOI of 100 PFU/cell at 37°C for 60 min at neutral pH,
fixed at 2 h p.i. and stained with DAPI and a fluorescent lipid dye (PKH26).
Figure 8. Vaccinia WR MV localizes within endocytic structures that are positive for CD98 in cells. (A-E) Imaging of vaccinia WR MV internalization kinetics into CD98-positive vesicles. CD98+/+ MEFs were incubated for 60 min at 4°C with FITC-conjugated anti-CD98 antibody (green) and WR-A4-mCherry (red) at an MOI of 20 PFU/cell. Cells were shifted to 37°C for 0 min (A) 5 min (B), 15 min (C), 30 min (D) or 45 min (E) and individually washed with acidic buffer to remove surface-bound antibody prior to fixation. The confocal images in each panel are enlarged views of the areas outlined by the white squares. Arrowheads point to the CD98-positive vesicles containing WR MV. (F) The percentage of MEF cells showing WR MV localizing in CD98-positive vesicles. In each time point, Z-sections of 60 cells were analyzed and the standard deviations are shown. (G) Percentage of WR MV particles per cell that localize within CD98-positive vesicles. The numbers of WR MV particles localizing in CD98-positive structures per cell and total WR MV particles per cell were quantified. In each time point, Z-sections of 60 cells were analyzed and the standard deviations are shown.

Figure 9. The CD98-positive endocytic structures are positive for phosphoinositol-3-phosphate (PI3P) and Rab5. (A). Co-localization of vaccinia MV-containing CD98+ vesicles with PI3P. CD98+/+ MEFs were incubated for 60 min at 4°C with FITC-conjugated anti-CD98 antibody (green) and WR-A4-mCherry (red) at an MOI of 20 PFU/cell, subsequently shifted to 37°C for 15 min and washed with acidic buffer to remove the surface-bound antibody. The cells were fixed and stained for intracellular PI3P-containing macropinosomes (cyan) as described in materials and methods. Arrowheads point to the MV-containing CD98+ vesicles that are positive for PI3P. The large insets in each panel are enlarged views of the areas outlined by the small squares. (B) Co-localization of vaccinia MV-containing CD98+ vesicles with an endosome marker Rab5. CD98+/+ MEFs were treated with anti-CD98 antibody (green) and WR-A4-mCherry (red) as described in A. The cells were fixed,
permeabilized and stained for an endosome marker Rab5 (cyan). Arrowheads point to the MV-containing CD98+ vesicles that are positive for Rab5.

**Figure 10.** The overall structure of CD98 is important for vaccinia MV endocytosis in MEF cells. (A) Schematic drawings of the chimeric constructs between CD98 (white) and CD69 (black) and the anticipated antibody recognition patterns. Cytop, cytoplasmic domain; TM, transmembrane domain; Extracell, extracellular domain. (B) Cell surface expression of the CD98/CD69 chimera proteins in CD98 knockout MEFs. CD98-/- MEFs stably expressing each of the CD98/CD69 chimera proteins were stained with mouse anti-human CD98 (CD98, C69T98E98, C98T69E98) or mouse anti-human CD69 (C69T98E69, C98T98E69, CD69) antibodies and analyzed by FACS. (C) Immunoblot analysis of the YFP fusion proteins that are fused with CD98/CCD69 chimera constructs and expressed in CD98-/- MEFs. The blot was probed with a mouse anti-GFP antibody to detect YFP expression. Mock represents the CD98-/- MEFs without transfection. The asterisks mark the expected sizes of each chimera constructs. (D) Expression of the CD98/CD69 chimera proteins in CD98 knockout MEFs did not increase viral early gene luciferase expression. The above-mentioned cells were infected with WR MV at an MOI of 5 PFU/cell for 60 min and harvested at 2h p.i. for viral early gene luciferase assays. Luciferase activities in CD98+/+ MEFs were used as 100% control. The experiments were performed three times and the standard deviations are shown. (E) Expression of the CD98/CD69 chimera proteins in CD98 knockout MEFs did not increase vaccinia viral core uncoating. The above-mentioned cells were infected with WR MV at an MOI of 40 PFU/cell for viral core uncoating assays as described above. In each group, at least 40 cells were counted to obtain an average number of viral cores/cell; viral core number obtained from CD98+/+ MEFs was used as 100% control. The experiments were performed three times and the standard deviations are shown. (F) Amino acid (aa) transport assay in...
CD98 knockout MEFs expressing CD98/CD69 chimera proteins. The above-mentioned cells were washed, pre-incubated in aa uptake solution and then pulsed for 1 min with 1 µCi/ml [14C] L-leucine as described in materials and methods. Cells were washed, lysed, and the radioactivity was measured by a liquid scintillation counter. The counts per minute (CPM)/mg protein in the lysate are shown. The experiments were performed five times and the standard deviations are shown.
**TABLE** 1  Quantification of lipid raft association of CD98 (4F2_human/ SLC3A2) in mock or virus-infected cells using four unique peptides with sequences shown in the right column.

<table>
<thead>
<tr>
<th>Relative abundance (infection/ mock)</th>
<th>No. of CD98 unique peptide</th>
<th>Unique CD98 peptide sequence</th>
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<td>1.75±0.06</td>
<td>4</td>
<td>GLVLGPIHK</td>
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TABLE 2 WR entry pathway comparison between HeLa cells and MEFs under different inhibitor treatments.

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<tr>
<th>Treatment/Inhibitor</th>
<th>HeLa</th>
<th>MEF</th>
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<tr>
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S. sensitive; R. resistant