The Cytoplasmic Domain of Rhesus Cytomegalovirus Rh178 Interrupts Translation of Major Histocompatibility Class I Leader Peptide-Containing Proteins prior to Translocation[▽]

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Cytomegalovirus (CMV) efficiently evades many host immune defenses and encodes a number of proteins that prevent antigen presentation by major histocompatibility complex class I (MHC-I) molecules in order to evade recognition and killing of infected cells by cytotoxic CD8⁺ T cells. We recently showed that rhesus CMV-specific Rh178 intercepts MHC-I protein translation before interference of MHC-I maturation by homologues of the human CMV US6 family. Here, we demonstrate that Rh178 localizes to the membrane of the endoplasmic reticulum, displaying a short luminal and large cytosolic domain, and that the membrane-proximal cytosolic portion is essential for inhibition of MHC-I expression. We further observed that Rh178 does not require synthesis of full-length MHC-I heavy chains but is capable of inhibiting the translation of short, unstable amino-terminal fragments of MHC-I. Moreover, the transfer of amino-terminal fragments containing the MHC-I signal peptide renders recipient proteins susceptible to targeting by Rh178. The cytosolic orientation of Rh178 and its ability to target protein fragments carrying the MHC-I signal peptide are consistent with Rh178 intercepting partially translated MHC-I heavy chains after signal recognition particle-dependent transfer to the endoplasmic reticulum membrane. However, interference with MHC-I translation by Rh178 seems to occur prior to SEC61-dependent protein translocation, since inhibition of MHC-I translocation by eeyarestatin 1 resulted in a full-length degradation intermediate that can be stabilized by proteasome inhibitors. These data are consistent with Rh178 blocking protein translation of MHC-I heavy chains at a step prior to the start of translocation, thereby downregulating MHC-I at a very early stage of translation.

Cytomegaloviruses (CMV), members of the betaherpesviridae, are masters at evading the host immune system. All CMV genomes dedicate many of their open reading frames (ORFs) to escaping various mechanisms of immune defense. CMV-encoded immunomodulators function to circumvent cell-autonomous defenses such as apoptosis and the interferon (IFN) response, as well as to prevent innate and adaptive immune responses by natural killer (NK) cells and T cells (5, 13, 34, 61). These proteins allow the virus to establish primary infection, maintain persistent infection, and support repeated superinfection of chronically infected hosts. The study of cytomegaloviral immunomodulatory proteins not only has underscored the important and delicate relationship between virus and host but also has revealed novel proteins of the host immune system like the UL16-protein binding family of NKG2D ligands and the UL18-binding NK cell inhibitory receptor LIR-1 (7, 8).

In addition, CMV immunomodulators have been employed to decipher basic cell biological principles such as protein quality control. Glycoproteins within the US6 family of human CMV (HCMV) (25) block endoplasmic reticulum (ER)-associated degradation of major histocompatibility complex class I (MHC-I) proteins and thereby prevent antigen presentation to CD8⁺ T cells. Specifically, US2 and US11 facilitate rapid retrotranslocation of MHC-I from the ER to the cytoplasm, fol-

lowed by proteasomal degradation (59, 60). Despite their similar type I transmembrane topology and luminal Ig-like folds (16), US2 and US11 achieve the endpoint of MHC-I dislocation from the ER by distinct mechanisms. US2-mediated retrotranslocation requires signal peptide peptidase (SPP), protein disulfide isomerase (PDI), and p97 ATPase (31, 35, 51). In contrast, US11 utilizes its TM domain to recruit Derlin-1 and Sel1L by a presumably independent yet complementary pathway (33, 38).

Additionally, US6 binds directly to the transporter associated with antigen processing (TAP) in the ER lumen (1, 21), causing a conformational change and subsequent inhibition of peptide loading and maturation of MHC-I heterodimers (22). US3 interferes with the functions of peptide loading complex chaperones tapasin (42) and protein-disulfide isomerase (43), thereby complementing US6 abrogation of MHC-I peptide loading and causing MHC-I retention within the ER. The rhesus CMV (RhCMV) genome contains a cluster of genes that encode functional homologues of the HCMV US2-11 region, with RhCMV Rh182, Rh184, Rh185, and Rh189 corresponding to HCMV US2, US3, US6, and US11, respectively (41). When the US2-11 region (including US2, US3, US6, and US11) is deleted from HCMV, MHC-I heavy-chain (HC) surface expression in infected cells reverts to steady-state levels. However, when we deleted the homologous region (Rh182 to Rh189) from RhCMV, MHC-I levels at the cell surface recovered only slightly, which led to the discovery of an RhCMV-specific mechanism of MHC-I inhibition, termed viral inhibition of heavy chain expression (VIHCE). The process of

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VIHCE was determined to be mediated by an RhCMV protein encoded by Rh178 (45).

Rh178 encodes for a 212-amino-acid (aa) protein that is ER localized and has no known homology to the US6 family of MHC-I inhibitors or any other viral or cellular protein. Interestingly, Rh178 seems to prevent HC expression by a unique posttranscriptional pathway. Based on the finding that Rh178 function is specifically dependent on the MHC-I signal peptide (SP) (45), we hypothesized that Rh178 specifically prevents early events during translation or translocation of MHC-I HC.

The HC of all MHC-I alleles consists of N-terminal cleavable SP, $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains, a transmembrane (TM) domain, and a short C-terminal cytosolic domain. The HC forms a heterodimer with $\beta 2$ -microglobulin (9). Like other type I membrane proteins, nascent MHC-I is targeted to the ER as the SP emerges from the ribosome, and it binds to the signal recognition particle (SRP) (17). As SRP binds to its receptor on the ER membrane, the SP is released, the ribosome is transferred to the SEC61 translocon, and the nascent chain is cotranslationally translocated into the ER lumen (39). Translocation is terminated by synthesis of the TM segment (stop transfer sequence) to establish a type I topology with the C-terminal domain residing in the cytosol. This interaction at the ER membrane allows translocation to be initiated and the nascent MHC-I protein to be fed through the Sec61 translocon, allowing the bulk of the protein to reside in the ER lumen, anchored only by the stop transfer sequence that becomes the C-terminal transmembrane domain (63). Despite the polymorphic nature of MHC-I molecules within and among different animal species, the SP is highly conserved (11, 55). The determination that Rh178 relies on the SP sequence for MHC-I downregulation indicates that RhCMV may take advantage of the conserved SP sequence among MHC-I alleles for immune evasion.

One obstacle in understanding the function of Rh178 is that it is poorly expressed in the absence of viral infection. To overcome this, we used a codon-optimized version that is highly expressed in transfected cells. Using an *in vitro* system, we also determined that Rh178 downregulates MHC-I HC during early stages of translation and showed that the MHC-I SP is not only necessary but also sufficient for VIHCE. In contrast to small molecule inhibitors that block MHC-I HC translocation, Rh178 acts prior to this step by inhibiting translation of the full-length protein.

MATERIALS AND METHODS

Cells, virus, and antibodies. HeLa-Tet-Off cells were obtained from Clontech, and telomerized rhesus fibroblasts (TRF) (6) were obtained from Jay Nelson. TRF-178 cells were made by cloning rh178-FLAG into pRevTre (Clontech), transfecting the resultant vector into Phoenix-AMPHO packaging cells (provided by Ashlee Moses), and infecting TRFs with the resulting retrovirus, followed by selection in 200 μ g/ml hygromycin (Invitrogen). HeLa cells, TRFs, and stable transfectant TRF-178 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. All cells were grown at 37°C in humidified air with 5% CO₂. Adenovirus containing the tet transactivator (Ad-tTA) was obtained from David Johnson. Mouse anti-Flag M2, anti-Flag-fluorescein isothiocyanate (FITC) conjugate, and mouse anti-HA (clone HA-7) antibodies were purchased from Sigma. Mouse anti-GAPDH (clone 6C5), mouse anti-integrin α V (clone P2W7), and goat anti-mouse-horseradish peroxidase (HRP) conjugate antibodies were purchased from Santa Cruz Biotechnology. Goat anti-mouse immunoglobulin/RPE antibody was purchased from Dako. Alexa Fluor 594 goat anti-

mouse and Alexa Fluor 647 chicken anti-mouse immunoglobulins (H+L) were purchased from Invitrogen. Anti-MHC class I monoclonal W6/32 antibody was purchased from Abcam.

Plasmid construction. Rh178 was originally PCR amplified from RhCMV strain 68-1 (provided by Scott Wong). It was Flag tagged and codon-optimized for expression in mammalian cells. Codon optimization services were provided by GeneArt. VIHCE-CFI and all VIHCE mutants were constructed by PCR and inserted into EcoRI and BamHI sites of the cloning vector pUHD10-1 (62). The sense primer for all mutants in this series is VIHCE-F, and the antisense primers for VIHCE-CFI, VIHCE Δ 10, VIHCE Δ 20, VIHCE Δ 30, VIHCE Δ 40, VIHCE Δ 50, VIHCE Δ 60, and VIHCE Δ 70 are, respectively, VIHCE-FI-R, Δ 10-R, Δ 20-R, Δ 30-R, Δ 40-R, Δ 50-R, Δ 60-R, and Δ 70-R. Sequences of all oligonucleotides are provided in Table 1. VIHCE TM SytII was cloned into BamHI and HindIII sites of pUHD10-1 by using three sense primers in sequence, TMS1-F, TMS2-F, and VIHCE3-F, and antisense primer TM-R. HLA-A3 truncations and A3-CD8 fusion constructs were also cloned into pUHD10-1 by PCR into EcoRI and BamHI sites. HLA-A3 truncations with C-terminal HA tags were each created with the same sense primer, A3-F. Antisense primers for A3-HA, A3 294, A3 206, A3 160, and A3 114 are A3-HA-R, A3 294-R, A3 206-R, A3 160-R, and A3 114-R, respectively. Control plasmid CD8-HA was created by inserting CD8 α from CD8 α -pRMHA (23) into the EcoRI and BamHI sites of pUHD10-1. Primers used for creation of CD8-HA were CD8-HA-R and CD8-F. HLA-A3-CD8 fusions and an Rh67-CD8 fusion were created by a triple ligation of HLA-A3 or Rh67 truncations, CD8-HA (without the CD8 signal sequence), and pUHD10-1. The HLA-A3/Rh67 and CD8-HA PCR products were fused with an added XbaI site. The CD8 sequence, minus SP, was generated by using primers CD8noSS-F and CD8-HA-R. A3 114, A3 73, A3 50, and A3 24 fragments were made using sense primer A3tr-F and antisense primers 114-R, 73-R, 50-R, and 24-R, respectively. The Rh67 fragment was made using sense primer Rh67-F and antisense primer Rh67-31-R. Rh67-FI was created with sense primer Rh67-FI-F and antisense primer Rh67-FI-R. SSFIA3 was created with sequential sense primers SSFIA3-F1, SSFIA3-F2, and SSFIA3-F3 and antisense primer SSFIA3-R. Table 1 shows a list of all oligonucleotides used in this study to create mutant versions of Rh178 and HLA-A3.

***In vitro* transcription, translation, and proteinase K digestion.** mRNA was transcribed with SP6 RNA polymerase (Promega) using 500 ng of plasmid DNA in a 10- μ l volume at 40°C for 1 h in reaction mixtures containing 40 mM Tris-HCl (pH 7.5), 6.0 mM magnesium acetate, 2 mM spermidine, 0.5 mM (each) ATP, CTP, and UTP (all from Promega), 0.1 mM GTP, 0.5 mM GpppG (Promega), 10 mM dithiothreitol (DTT), 0.75 U/ml RNase inhibitor (Promega), and 0.4 U/ml SP6 RNA polymerase. Rabbit reticulocyte lysate (RRL) was prepared as previously described (49, 50). Translation was performed at 25°C for 1.5 h in reaction mixtures containing 20% transcript mixture and 40% nuclease-treated RRL, additionally with 1 mM ATP, 1 mM GTP, 12 mM creatine phosphate (Promega), 40 μ M (each) 19 essential amino acids except methionine (Promega), 1 μ Ci/ μ l of ³⁵S-label (Express Protein labeling mix; Perkin-Elmer), 40 μ g/ml creatine kinase (Promega), 0.2 U/ μ l RNase inhibitor (Promega), 10 mM Tris-HCl (pH 7.5), 100 mM potassium acetate, 2 mM DTT, and 2 mM MgCl₂. Canine pancreas microsomal membranes (0.3 μ l) (Promega) were added at the start of translation. For protease protection experiments, proteinase K (Fermentas) was added (2 mg/ml) in the presence or absence of 1% Triton X-100. Samples were incubated at 4°C for 1 h, and residual protease was inactivated by rapid mixing with phenylmethylsulfonyl fluoride (10 mM) and heating to 100°C in 10 volumes of 1% SDS–0.1 M Tris, pH 8.0, for 5 min. Samples were then added directly to SDS loading buffer, electrophoresed on a 16.5% Tris-Tricine gel, and visualized by autoradiography.

Transfection and nucleofection. For expression and cotransfection experiments, 500 ng VIHCE mutants was transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. VIHCE mutants, HLA-A3 truncations, and HLA-A3-CD8 fusions were electroporated into telomerized rhesus fibroblasts (TRFs) (6) using the AMAXA Nucleofector II (AMAXA Biosystems) and cell line solution L (Lonza AG). Plasmid DNA (500 ng to 2 μ g) was mixed with 0.5×10^6 to 2×10^6 TRF, suspended in 100 μ l AMAXA solution, and electroporated under parameters defined by program T-030. Cells were then recovered in 500 μ l RPMI (Gibco) for 45 min at 37°C, followed by plating in complete DMEM. Cotransfection with green fluorescent protein (GFP) vector revealed that transfection efficiency was consistently between 50 and 80%.

SLO and immunofluorescence. HeLa cells were plated to ~70% confluence in 24-well dishes on coverslips and either mock transfected (500 ng control plasmid) or transfected with 500 ng C- or N-Flag-tagged Rh178. Streptolysin O (SLO) permeabilization and immunofluorescence were performed 48 h posttransfection as described previously (47). Briefly, SLO (1 μ g/ml) is preactivated by incubating

TABLE 1. Oligonucleotides used to create mutant constructs

Oligonucleotide	Sequence (5'–3')
VIHCE-F	CTGGAATTCATGCTGCTCTAC
VIHCE-FI-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCCAGGGCCTTGGGGGGGGCT
Δ10-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGGGGCTGGAGTCGTGGTA
Δ20-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGGGGCTGGAGTCGTGGTA
Δ30-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGCCGGACAGGGCGGAGTC
Δ40-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGTCGCTGGGGGGTCTGGG
Δ50-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCTCTGGAGCCCTGGGCAGC
Δ60-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGTCGACGACGAGGAGTG
Δ70-R	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGGAGGAGGAGGAGTCCGGC
TMS1-F	GGGCTCCTGCTTCTACCTGCTGCTTCTGCATCTGCGAGTTCTGCAGGTGC
TMS2-F	TTCTTCAGGGCACTGATCGCCATTGCTGTGGTTGCTGGGGCTCCTGCTTCTC
TMS3-F	CTGGGATCCTCATCACTTGTGTCGTCGTCGTCCTTGTAGTCGTCGACCTTCTTCAGGGCACTG
TM-R	CTGAAGCTTAGTCATCACTTGTGTCGTC
A3-F	CTGGAATTCATGGCCGTCATGGCGCCCCGAAC
A3-HA-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTCATGGGTACACTTTACAAGCTGTG
A3 294-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTCATGGGTAGAGGGGCTGGGCAGAC
A3 206-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTCATGGGTACGTCGCGCTGCAGCGTC
A3 160-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTCATGGGTACGCCGCGGTCCAAGAGC
A3 114-R	GTCGGATCCCTACTAGGCGTAGTCTGGCACGTCGTCATGGGTAGGCCTCGCTCTGGTTGTAG
A3tr-F	CTGGAATTCGCGCCACCATTGGCCGTCATGGCGCCC
114-R	CTGTCTAGAGCCCTCGCTCTGGTTGTA
73-R	CTGTCTAGACGCCCGCGGCTCCATCCT
50-R	CTGTCTAGAGCCACGCGCATGAAGCG
24-R	CTGTCTAGACGCCAGGTCTGGGTCAG
CD8-F	CTGGAATTCGCGCCACCATTGGCCCTTACCAGTGACC
CD8noSS-F	CTGTCTAGAAGCCAGTTCCGGGTGTCG
CD8-HA-R	CTGGGATCCTCAAGCGTAATCTGGAACATCGTCATGGGTAGACGTATCTCGCCGAAAG
Rh67-F	CTGGAATTCGCGCCACCATTGCTGCTCAGCGTGGCG
Rh67-31-R	CTGTCTAGATACCCCAAAGCCACCGT
Rh67-FI-F	CTGGAATTCATGCTGCTCAGCGTGGCGATGGTG
Rh67-FI-R	CTGGGATCCTCACTTGTGTCGTCGTCGTCCTTGTAGTCTGGAATGGTTATCATTTTC
SSFA3-F1	GACTACAAGGACGACGACGACAAGGGCTCCCACTCCATG
SSFA3-F2	CTGCTACTCTCGGGGGCCCTGGCCCTGACCCAGACCTGGCGGACTACAAGGACGAC
SSFA3-F3	CTGGAATTCATGGCCGTCATGGCGCCCCGAACCCTCCTCTGCTACTCTCGGGG
SSFA3-R	CTGGGATCCTCACACTTTACAAGCTGT

at 37°C for 10 min with 4 mM DTT (Fisher Scientific) in a sodium-free buffer (25 mM HEPES, 2.5 mM MgCl₂, 25 mM KCl, 25 mM sucrose, pH 7.4). Activated SLO was then added to the cells at 4°C for 10 min. Cells were washed 2× with cold sodium-free buffer and then permeabilized at 37°C for 15 min, followed by fixation in 1% formaldehyde (Fisher Scientific) at room temperature (RT) for 10 min. Cells were blocked with 1% bovine serum albumin (Fisher Scientific) and 5 mg/ml glycine (Fisher Scientific) in sodium-free buffer at RT for 15 min. Cells were then incubated for 1.5 h with a 1:200 dilution of anti-Flag antibody in either sodium-free buffer (for SLO-permeabilized cells) or 0.25% saponin (Calbiochem) in sodium-free buffer (for saponin-permeabilized cells). Cells were rinsed 3× with sodium-free buffer and then incubated for 1 h with a 1:500 dilution Alexa Fluor 594 goat anti-mouse antibody. Cells were rinsed 3× with sodium-free buffer and then mounted onto glass slides (Fisher Scientific) with VECTASHIELD (Vector Laboratories). Slides were visualized on a Zeiss Axioskop 2 Plus fluorescence microscope, and images were produced with AxioVision v4.6 software (Zeiss).

Immunoblotting. HeLa cells were harvested 48 h posttransfection or AMAXA nucleofection and lysed directly in 2× Laemmli buffer (28). Samples were run through QIAshredder columns (Qiagen) to decrease viscosity, and 2-mercaptoethanol (Sigma) was added to 5%. Samples were separated on 10% polyacrylamide gels by SDS-PAGE (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking for 30 min in 10% powdered milk and PBS–0.1% Tween 20 (Fisher Scientific) (PBST), membranes were incubated with primary antibodies directed against either Flag (1:5,000) or HA (1:2,000) epitope tags, GAPDH (1:10,000), or integrin αV (1:5,000) in 5% milk in PBST. Membranes were washed 3× with PBST and then incubated with secondary antibody goat anti-mouse HRP (1:5,000) in PBST. After washing 3× with PBST, membranes were incubated with Supersignal West Pico chemiluminescent substrate (ThermoScientific) for 5 min and developed on chemiluminescent film (GE Healthcare).

Flow cytometry. To monitor surface expression of MHC-I in Rh178-positive cells, TRFs were harvested 48 h after AMAXA nucleofection, trypsinized, and

resuspended in 10% fetal bovine serum (FBS) (HyClone)/PBS. Cells were washed 2× with PBS, followed by a 30-min incubation with a 1:500 dilution of W6/32 antibody. Cells were washed 3× with PBS and then incubated with a 1:500 dilution of Alexa Fluor 647 chicken anti-mouse antibody for 30 min in the dark. Cells were then fixed in 2% paraformaldehyde (Electron Microscopy Sciences) at RT for 15 min and permeabilized with 1% wt/vol saponin in 10% FBS–PBS to allow for intracellular staining. Cells were then incubated with a 1:200 dilution of anti-Flag M2 FITC-conjugated antibody at RT for 30 min, washed 3×, and resuspended in PBS. All antibody incubation and wash steps were performed at 4°C. Surface expression of MHC-I and intracellular expression of Rh178 were quantified using flow cytometry (FACSCalibur; BD Biosystems). Data analysis was performed using FlowJo software v7.6 (Treestar Inc.).

Eeyarestatin treatments. Eeyarestatin 1 (ES₁) and eeyarestatin R35 (ES_{R35}) were kind gifts from Stephen High (University of Manchester, Manchester, United Kingdom). Eeyarestatin treatments were based on methods previously described (10). TRFs in culture were treated with dimethyl sulfoxide (DMSO) or 10 μM of the proteasome inhibitor MG132 for 4 h, followed by treatment with DMSO, 10 μM ES₁, or 10 μM ES_{R35} for 1 h in the continuing presence of DMSO or MG132. Subsequently, cells were starved for 30 min in methionine- and cysteine-free medium in the presence of previously applied compounds, followed by 30 min of metabolic labeling in the presence of drugs with ³⁵S-label (Express Protein labeling mix; Perkin-Elmer). Cells were rinsed three times in PBS and either collected and lysed or chased for 90 min before collection. Samples were lysed in PBS–1% NP-40–protease inhibitor cocktail (Halt protease inhibitor cocktail; Thermo Scientific) for 30 min. All steps were performed at 4°C. Samples were then pelleted for 10 min at 16,100 rpm, precleared with Protein A/G Plus Sepharose beads (Santa Cruz) for 30 min, and incubated with primary antibody (W6/32 and HC-10) for 1 h, followed by the addition of Protein A/G Plus Sepharose beads and incubation for 1 h. Bound samples were washed three times in PBS and eluted with 2× Laemmli buffer, followed by SDS-PAGE and autoradiography.

FIG. 1. Rh178 is a type Ib ER-resident transmembrane protein. (A) Kyte-Doolittle hydropathy plot of Rh178. Indexes above 1 (dashed line) represent hydrophobic or transmembrane domains. VIHCE is represented below the x axis of the plot, and the most likely transmembrane domain (TM) and another possible transmembrane or membrane-associated domain (?) are represented by black squares. (B) HeLa cells were either mock transfected or transfected with a vector containing an N- or C-terminal Flag tag. After 48 h, cells were treated either with saponin, to permeabilize all cellular and organelle membranes, or with streptolysin O (SLO) to permeabilize only the cell membrane. Immunofluorescence was performed to visualize the Flag-tagged Rh178 with anti-Flag epitope tag antibody M2, followed by an Alexa Fluor anti-mouse 547 antibody. Nuclei are stained with VectaShield (blue). (C) mRNAs were translated in rabbit reticulocyte lysate (RRL) in the presence of canine microsomal membranes, and the resultant proteins were digested with proteinase K and analyzed by SDS-PAGE and autoradiography. pPI, preprolactin control. (D) Diagram showing the predicted orientation of Rh178.

RESULTS

Rh178 is a type I transmembrane protein that is anchored in the ER membrane and faces the cytosol. Our previous experiments determined that Rh178 is localized in the membrane of the endoplasmic reticulum (ER) (45), but the number of transmembrane domains and the orientation within the ER membrane were unknown. A Kyte-Doolittle hydropathy plot (27) indicates one highly likely transmembrane domain close to the N terminus and at least one other highly hydrophobic region that represents a potential transmembrane or membrane-associated domain (Fig. 1A). To determine the membrane orientation of Rh178 we used a codon-optimized version of Rh178 to improve expression in tissue culture cells. HeLa cells were transfected with a control plasmid or with C- or N-terminal Flag-tagged versions of Rh178 (Rh178-Cfl and Rh178-Nfl, respectively), followed by immunofluorescence of saponin- or streptolysin O (SLO)-treated cells. Saponin permeabilizes all cellular membranes, whereas SLO permeabilizes only the plasma membrane. As expected, the Flag epitope tag was detected in both Rh178-Cfl and Rh178-Nfl transfected cells upon saponin treatment (Fig. 1B). In SLO-treated cells, only Rh178-Cfl was detectable, indicating that the C terminus of Rh178 extends into the cytosol, whereas the N terminus of Rh178 is not cytosolically accessible. This result indicates that

the N terminus of Rh178 is in the lumen and the C terminus is in the cytosol. Therefore, Rh178 must possess an odd number of transmembrane domains. Based on the hydropathy plot, the likelihood of three or more transmembrane domains is low, leaving a single transmembrane protein as the most likely orientation for Rh178. The hydrophobic domain near the N terminus likely functions as a type I signal anchor to translocate the N terminus and span the membrane. To confirm this hypothesis we performed a proteinase K protection assay of 35 S-labeled Rh178 translated *in vitro* in the presence of microsomal membranes. Proteinase K (PK) digests portions of proteins outside the microsomes, whereas protein domains inside the microsomes are protected. For control, we used preprolactin (pPI) and HLA-A3, which both undergo SP cleavage (2). As expected, only cleaved prolactin, which is found inside the microsomes, was protected from PK digestion, whereas the cytosolic precursor was degraded in the presence of PK (Fig. 1C). HLA-A3 spans the microsomal membranes with only a short extramicrosomal portion, so the majority of HLA-A3 was also protected from PK. The small shift in size resulted from digestion of the short cytoplasmic tail (Fig. 1C). In contrast, almost all of Rh178 was digested by PK, leaving a small ~6-kDa fragment (Fig. 1C), demonstrating that most of the protein resides outside the microsomal lumen. Taken together,

FIG. 2. Membrane and membrane-proximal domains of Rh178 are important for downregulation of MHC class I. (A) Diagrams of Rh178 C-terminal deletions, each containing a C-terminal Flag tag. The transmembrane domain of Rh178 was replaced with the transmembrane domain of synaptotagmin II (TM SytII). (B) HeLa cells were either mock transfected or transfected with the Rh178 mutants. After 48 h, cells were lysed in an SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-Flag epitope tag antibody M2 followed by an anti-mouse HRP. (C) Analysis of the Rh178 downregulation of MHC class I. Rh178 mutants were electroporated into telomerized rhesus fibroblasts (TRFs) using the AMAXA nucleofection system. Flow cytometry was used to quantify cell surface expression of total MHC class I in Flag-negative and Flag-positive cell populations. Flow cytometry using anti-Flag antibody was conjugated to FITC-separated Flag-negative and -positive populations. Surface staining with MHC class I antibody W6/32 was followed by anti-mouse APC antibody to quantify surface MHC class I expression. Each panel demonstrates the mean fluorescence intensity of Flag-negative cells (black line) compared to that of the Flag-positive cells (dotted line). (D) Quantification of cell surface MHC class I expression in Flag-negative [Flag(-)] and Flag-positive [Flag(+)] cells.

these results indicate that Rh178 is a single-spanning transmembrane protein with a type I topology (Fig. 1D).

Membrane-proximal domains of Rh178 are indispensable for VIHCE. The type I topology of Rh178 suggests a prominent role of the cytosolic domain for VIHCE. BlastP searches with the C-terminal portion of Rh178 did not reveal homology to any known protein. We therefore mapped the determinants within the C terminus of Rh178 that were required for VIHCE by constructing a series of C-terminal deletion mutants that contained a C-terminal Flag epitope tag (Fig. 2A). To test whether the transmembrane (TM) domain of Rh178 is required for VIHCE, we replaced the TM domain with that of another type I TM protein, synaptotagmin II (26) (TM SytII) (Fig. 2A). Expression in HeLa cells demonstrated that the

corresponding proteins had the expected molecular weight and were expressed at similar levels (Fig. 2B). Immunofluorescence of transfected HeLa cells further confirmed equivalent expression of all constructs (data not shown). To examine the ability of these constructs to downregulate MHC-I, we used the AMAXA nucleofection system to express each Rh178 mutant in telomerized rhesus fibroblasts (TRFs). By gating for Flag-positive cells using flow cytometry, we compared MHC-I surface expression of Rh178-negative and Rh178-positive cell populations (Fig. 2C). Compared to nontransfected cells, MHC-I surface levels were significantly reduced in TRFs expressing full-length Rh178. A similar degree of MHC-I downregulation was observed for Δ10, Δ20, and Δ30. Cells expressing Δ40 showed a slight downregulation of MHC-I, indicating

some residual activity. However, MHC-I levels were unchanged in cells expressing Rh178 lacking 50 amino acids or more. These results indicate that the membrane-distant 30 amino acids of Rh178 are not essential for VIHCE. Cells expressing the TM SytII construct showed only a minor reduction of MHC-I surface levels, indicating that, in addition to serving as a membrane anchor, the TM domain also contributes to VIHCE. Quantification of these data is demonstrated in Fig. 2D. We also constructed a mutant lacking the entire N-terminal TM and luminal domain. However, the resulting protein was highly unstable (data not shown). These data demonstrate that the TM domain and membrane-proximal cytoplasmic domain represent the functional core of Rh178 for VIHCE.

Rh178 downregulates truncated versions of HLA-A3. We previously demonstrated that replacement of the MHC-I SP with an unrelated SP renders MHC-I resistant to Rh178 (45). This finding strongly suggested that Rh178 requires the translation of the MHC-I SP. However, despite this circumstantial evidence that MHC-I is translated, we cannot detect a translation or degradation intermediate of the MHC-I HC, even in the presence of proteasome inhibitors (45). Thus, we hypothesized that only a very short translation product is generated in the presence of Rh178 followed by rapid, proteasome-independent degradation. One corollary of this hypothesis is that Rh178 should prevent expression of very short amino-terminal fragments of MHC-I. To test this assumption, we created a series of C-terminally truncated versions of HLA-A3, each named for the number of amino acids remaining and containing a C-terminal HA epitope tag to facilitate detection by Western blotting (Fig. 3A). The human MHC-I allele was chosen for these experiments because we have previously shown that HLA-A3 expression is inhibited by Rh178 in an SP-dependent manner (45). Like all classical MHC-I molecules, HLA-A3 is comprised of a signal sequence and $\alpha 1$, $\alpha 2$, and $\alpha 3$ domains, in addition to a C-terminal transmembrane domain and short cytoplasmic tail. While the longer constructs were stably expressed upon transfection, the shortest construct, A3 114, was rapidly degraded unless proteasome inhibitors were added (Fig. 3B). To determine whether expression was inhibited by Rh178, we generated a stable cell line under the control of the tet-off system (TRF-178) (18), in which Rh178 is induced upon transduction with an adenovirus expressing the tetracycline-responsive transcriptional activator (Ad-tTA) (Fig. 3B). Using this system, we compared VIHCE for each of the HLA-A3 constructs with or without induction of Rh178. Upon expressing Rh178 by Ad-tTA transduction, significantly reduced levels of full-length HLA-A3 were observed (Fig. 3B). Interestingly, expression of all other HLA-A3 truncation mutants was also inhibited upon induction of Rh178 (Fig. 3B). Moreover, even the shortest fragment, A3 114, was inhibited in the presence of Rh178 even though proteasome inhibitors were needed to see any expression at all. Expression of HLA-A3 fragments that were shorter than 114 amino acids could not be stabilized by proteasome inhibitors (data not shown). From these data, we conclude that Rh178 is able to inhibit MHC-I translation prior to completion of fewer than the first 114 amino acids.

The signal peptide of HLA-A3 is sufficient for VIHCE. Since the N-terminal 114 amino acids of HLA-A3 were sufficient for

FIG. 3. Rh178 downregulates truncated versions of HLA-A3. (A) Diagram of C-terminal HLA-A3 mutants. A3 294 lacks the transmembrane domain and C terminus, A3 206 lacks the $\alpha 3$ domain, A3 160 lacks the $\alpha 3$ and half of the $\alpha 2$ domain, and A3 114 lacks the $\alpha 3$ and $\alpha 2$ domain. All constructs contain a C-terminal HA epitope tag. (B) Truncated HLA-A3 constructs are downregulated by Rh178. TRF-178 is a telomerized rhesus fibroblast cell line that stably expresses VIHCE under the control of the tet-off system. Control adenovirus (Ad-RTA) (–) or adenovirus with the tet-transactivator (Ad-tTA) (+) was added at a multiplicity of infection (MOI) of 5 to TRF-178, followed by AMAXA nucleofection of truncated constructs of HLA-A3 24 hours postinfection (h.p.i.). Cells were lysed 72 h.p.i. in SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-HA epitope tag antibody, followed by anti-mouse HRP antibody. Blots were also probed with anti-Flag epitope tag antibody M2 and anti-GAPDH as a loading control. MG132 was added to stabilize the A3 114 construct, which was not expressed well in the absence of MG132.

VIHCE, we wondered whether their transfer to another protein would confer susceptibility to inhibition by Rh178. To address this question, we replaced the N-terminal SP of CD8 with the N-terminal 114 amino acids of HLA-A3. Upon transfection into TRF-178 cells, HA-tagged full-length CD8 was not affected by the induction of Rh178 (Fig. 4B). Similarly, expression of endogenous protein integrin $\alpha 5$ was not inhibited by Rh178 (Fig. 4C). In contrast, expression of the A3 114-CD8 chimeric protein was strongly reduced upon the induction of Rh178. Because HLA-A3 protein products with fewer than 114 amino acids could not be stabilized by MG132, this finding allowed us to further narrow down the minimal sequence required for VIHCE. Therefore, we fused even shorter portions of the HLA-A3 N terminus to the CD8 molecule, including a construct in which only the first 24 amino acids, comprising only the leader peptide of HLA-A3, were transferred (Fig. 4A). Interestingly, expression of all of the shorter HLA-A3-CD8 fusions was also inhibited by Rh178 (Fig. 4B). This result was surprising, since we previously observed that expression of a chimeric protein of CD4 with the HLA-A3 SP was not inhibited in RhCMV-infected TRFs (45). A possible explanation is that expression levels of Rh178 are lower in virally infected cells than upon transfection of a codon-optimized gene product. The increased expression level achieved by codon optimization might be required to prevent the translation of highly expressed artificial constructs such as CD8 or CD4 fusion proteins. Nevertheless, these data clearly demonstrate that the SP is not only necessary but also sufficient for VIHCE.

FIG. 4. The signal peptide of HLA-A3 is sufficient for Rh178-directed downregulation. (A) Diagram of A3-CD8 fusions were created by replacing the signal peptide of CD8 with an N-terminal portion of HLA-A3. The smallest fusion, A3 24-CD8, is a direct swap of the CD8 signal peptide with the HLA-A3 signal peptide. Each fusion mutant contains a C-terminal HA epitope tag. (B) A3-CD8 fusions are downregulated by Rh178. Control adenovirus (Ad-RTA) (–) or adenovirus with the tet-transactivator (Ad-tTA) (+) was added at a multiplicity of infection (moi) of 5 to TRF-178, followed by AMAXA nucleofection of A3-CD8 fusions 24 hours postinfection (h.p.i.). Cells were lysed 72 h.p.i. in SDS loading buffer and resolved by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-HA epitope tag antibody, followed by anti-mouse HRP antibody. Blots were also probed with anti-Flag epitope tag antibody M2 and anti-GAPDH as a loading control. (C) Endogenous integrin α5 is not downregulated by Rh178. Levels of integrin α5 were examined by immunoblot with anti-integrin αV antibody followed by anti-mouse HRP.

The UL40 homologue Rh67 is resistant to VIHCE. To further narrow down the target sequences required for VIHCE, we took advantage of another RhCMV protein, Rh67, which has an N-terminal predicted SP with homology to the HLA-A3 SP. In HCMV, the glycoprotein UL40 contains a 9-mer peptide within its SP that is highly similar to 9-mer peptides within the SP of MHC-I molecules. These SP-derived peptides are loaded into the binding groove of nonpolymorphic HLA-E, thereby upregulating surface HLA-E and engaging inhibitory CD94/NKG2A natural killer (NK) cell receptors (52). Thus, UL40 mimics the normal function of peptides derived from MHC-I SPs to prevent NK cell lysis of infected cells in which the classical, polymorphic MHC-I molecules were destroyed by HCMV (56). Rh67 likely represents the functional homologue of UL40, since its predicted SP contains a 9-mer peptide (VMAPRTLIL) that differs by only one amino acid from the UL40 9-mer (VMAPRTLIL), whereas the remaining protein is not conserved. Interestingly, the Rh67 9-mer is identical to 9 amino acids within the HLA-A3 SP that is sufficient for Rh178-mediated downregulation of MHC-I (Fig. 5A). This homology raised the interesting question of whether Rh67 would be susceptible to VIHCE and gave us the opportunity to further map the susceptible sites recognized by VIHCE. However, expression of Rh67 was not affected upon cotransfection with Rh178 into HeLa cells, unlike expression of a Flag-tagged version of HLA-A3 (ssFlagA3), which, as expected, was downregulated by Rh178 to near control levels (Fig. 5B). To further confirm that the Rh67 SP is not sufficient to convey VIHCE susceptibility to CD8, we created a fusion of the Rh67 SP and CD8, tagged with HA (Rh67-CD8). In Rh178-expressing cells, there was no downregulation of Rh67-CD8. These results suggest that sequences outside this conserved 9-mer region are targeted for VIHCE. Moreover, these results are consistent with Rh67 potentially supporting expression of MamuE, the highly conserved rhesus homologue of HLA-E, despite viral interference with MHC-I expression by Rh178 as well as US6 family proteins.

VIHCE occurs prior to HC translocation. The finding that the MHC-I SP is sufficient for VIHCE implied that Rh178 targets the nascent HC after initiation of translation but before translation is completed. Translation of SP-containing proteins

FIG. 5. The RhCMV UL40 homologue Rh67 is not targeted by Rh178. (A) Alignment of the SP of HLA-A3 and Rh67 SP, 24 and 31 amino acids in length, respectively. The two 9-amino-acid stretches of exact homology are underlined. (B) Full-length Rh67 is not targeted by Rh178. HeLa cells were cotransfected with untagged Rh178 and either ssFlagA3 or Rh67-Fl. Cells were harvested 48 h posttransfection and stained intracellularly for Flag expression. (C) Rh67-CD8 fusion is not downregulated by Rh178. Nucleofection of Rh67-CD8 into TRF-178 cells was performed as described in the legend to Fig. 4B, followed by immunoblot for HA, Flag, or GAPDH.

is coupled to translocation via the signal recognition particle (SRP), which directs the ribosome-nascent chain complex (RNC) to the SRP receptor (SR) followed by transfer to the SEC61 translocon (46). Since Rh178 is a cytosol-facing, ER-associated transmembrane protein, it is reasonable to assume that Rh178 can only interfere with HC translation once the SRP has directed the RNC to the ER membrane. Thus, Rh178 could inhibit (i) the interaction of the SRP/RNC with the SR, (ii) the transfer of the RNC to the SEC61 translocon, or (iii) SEC61-mediated HC translocation across the ER membrane. Each of these steps could presumably prevent the completion of HC translation, thus resulting in rapidly degraded translation intermediates.

Recently, the small molecule inhibitor eeyarestatin 1 (ES_1) was shown to efficiently inhibit protein translocation and prevent transfer of the RNC from the SR to SEC61 (10). In contrast, ES_1 did not prevent docking of the SRP/RNC to the SR. Since Rh178 seems to interfere at a similar step with HC translation/translocation, we wondered whether the effect of ES_1 on HC translation would be similar to that of Rh178. TRFs were treated with DMSO, 10 μ M ES_1 , or the inactive analog ES_{R35} prior to metabolic labeling and immunoprecipitation of endogenously expressed MHC-I. Compared to that of DMSO-treated cells, recovery of MHC-I was strongly reduced in the presence of ES_1 , whereas ES_{R35} did not inhibit MHC-I translation (Fig. 6A). In contrast, MHC-I was immunoprecipitated from lysates obtained from TRFs treated with proteasome inhibitor MG132 prior to and during ES_1 treatment (Fig. 6A). Thus, it seems that inhibiting RNC transfer to SEC61 and SEC61-dependent protein translocation does not prevent the translation of full-length HC which is then degraded by the proteasome. In contrast to ES_1 -treated cells, cells that express Rh178 have no restoration of MHC-I expression even in the presence of MG132 (Fig. 6B and reference 45). Taken together, these data suggest that Rh178 acts upstream of the inhibitor ES_1 , possibly by preventing the docking of SRP/nascent HC complex to the SR.

DISCUSSION

Among the many host immune defense mechanisms counteracted by CMV (54) the interference with MHC-I-dependent antigen presentation and subsequent subversion of CD8⁺ T cell recognition is particularly complex and multifaceted, involving multiple genes in every CMV species that has been studied so far (3). In addition to their importance for immune evasion *in vivo* (20), mechanistic studies of US2, US3, US6, and US11 have given us important insights into basic cell biological principles (44). Here we explore a novel mechanism of interference with MHC-I expression by Rh178, which our previous observations suggest is a non-US6-related, RhCMV-specific protein that interferes with MHC-I expression in an SP-dependent manner (45). US2 and US11 and their RhCMV homologues downregulate MHC-I by redirecting assembled immature complexes to the cytosol, where they are degraded by the proteasome. US3 and US6 block peptide loading of MHC-I in the ER by interfering with tapasin and TAP, respectively. Thus, all of the US6-related proteins exert their effects after MHC-I has been successfully translated and translocated into the ER lumen. This posttranslational interference mech-

FIG. 6. Rh178 acts at a different stage of translation than small molecule inhibitor eeyarestatin. (A) TRFs were treated with either DMSO or MG132 for a total of 6 h. DMSO, ES_1 , or ES_{R35} was applied for 1 h, followed by a 30-min starvation period and a 30-min metabolic label, all in the presence of drugs as indicated. Cells were collected and lysed immediately and subjected to MHC-I immunoprecipitation, SDS-PAGE, and autoradiography. Bands were quantified using ImageJ, with DMSO-treated MG132 negative samples set to 1. (B) The AMAXA system was used to nucleofect TRFs with Rh178-Flag, in the absence or presence of MG132. Flow cytometry was used to quantify cell surface expression of endogenous MHC-I in Flag-negative and Flag-positive cell populations. Each panel demonstrates the mean fluorescence intensity of Flag-negative cells (black line) compared to that of the Flag-positive cells (dotted line).

anism is also reflected in the membrane topology of the US6 family since all members are type Ia TM proteins, comprised of a large ER luminal portion, a single TM domain, and a short cytoplasmic tail (16, 19, 32, 37). While TM domains and the short cytoplasmic portion have been implicated in the function of some US6 family members (30, 32), the large luminal domains are thought to be responsible for their substrate specificity, i.e., their ability to directly interact with MHC-I alleles or with components of the peptide loading complex (1, 16, 30, 32, 37). In stark contrast to the findings for US6 family members, the data presented here clearly show that Rh178 has a type Ib orientation, and that the bulk of this 212-amino-acid protein is cytoplasmic. Functional analysis of C-terminal deletion mutants of Rh178 demonstrates the importance of its cytosolic, membrane-proximal cytoplasmic core. In contrast, an Rh178 mutant with a substituted TM domain retained some functionality, indicating a less important role for the TM domain. Taken together, these data indicate that Rh178 intercepts nascent HC earlier than any other CMV-encoded MHC-I inhibitor and likely exerts this control at the cytosolic face of the ER membrane.

The cytosolic orientation of the functional part of Rh178 also implies that, unlike US6-related proteins, Rh178 should be able to recognize its substrate, the HC, prior to its complete

FIG. 7. Proposed model for Rh178-mediated VIHCE activity. (A) In the absence of Rh178, translation and translocation of MHC-I occur as normal. The signal peptide of the nascent chain emerges from the ribosome and is recognized by SRP, which guides the entire complex to the SRP receptor in the ER membrane. The ribosome-nascent chain complex is transferred to the SEC61 translocon, and the SRP is released back into the cytosol. MHC-I is translocated cotranslationally in a vectorial manner from the continuous pore formed by the ribosome and the translocon, and the signal peptide is cleaved in the ER lumen. Once MHC-I is fully translated, the ribosome subunits are released into the cytosol. (B) Rh178 is a type Ib ER-transmembrane protein that blocks this process very early in translation. This blockage is dependent on the MHC-I signal peptide and occurs before translocation. Whatever portion of MHC-I is made before translational arrest is likely degraded quickly in the cytosol.

translation. This hypothesis is strongly supported by our studies of progressively truncated versions of HLA-A3. We observed that Rh178 successfully interfered with the expression of even the shortest N-terminal fragment of HLA-A3 that we were able to express, a 114-amino-acid (aa) fragment that was detectable only in the presence of proteasome inhibitors. Thus, Rh178 clearly does not need complete MHC-I translation to recognize its substrate. Strikingly, chimeric proteins containing only the 24-aa SP of HLA-A3 fused to the N terminus of CD8 were targeted by Rh178, confirming that Rh178-mediated VIHCE is dependent upon the HLA-A3 SP. Since the native CD8 SP was not affected, we conclude that Rh178 is able to discriminate between HC-derived and non-HC-derived SPs. Additionally, Rh178 can distinguish between highly related SPs. The SP of Rh67, which contains 9 aa identical to 9 aa in the HLA-A3 SP, was not inhibited by Rh178. Since there is strong conservation of SP among MHC-I alleles (probably partially due to selective pressure by HLA-E), RhCMV has

thus identified an “Achilles heel” of the MHC-I pathway. However, because SP conservation is not perfect and since rhesus macaques have a more complex MHC-I locus than humans, with up to 10-fold higher sequence divergence (11), it is still possible that Rh178 preferentially targets some but not all MHC-I alleles, but this possibility requires further investigation.

Attempts to express even shorter fragments of HLA-A3 (73 aa) failed even when proteasomal degradation was inhibited (data not shown), suggesting that HC-derived polypeptides shorter than ~100 aa are degraded by a proteasome-independent process (assuming that the SPase is acting on such a truncated product, the resulting polypeptide could even be further shortened by ~25 aa). Similarly, it is possible that we are unable to detect prematurely truncated HC translation intermediates even in the presence of proteasome inhibitors due to proteasome-independent degradation. Indeed, short cytosolic peptides are known to be rapidly degraded by a num-

ber of proteases, e.g., amino-peptidases, tripeptidyl-peptidase 2, thimet oligopeptidase, dipeptidyl-peptidase-4, and others (12, 36, 48, 53). Therefore, we propose that Rh178 targets the nascent HC after the SP emerges from the ribosome and the nascent HC is targeted to the ER membrane by SRP. The length of the HC at this point of the translation process is estimated to be approximately 50 aa, based on earlier *in vitro* observations with preprolactin (40). Importantly, binding of the SRP to nascent polypeptide chains slows down translation (29). Thus, a possible mechanism for VIHCE would be that Rh178 prolongs this translational arrest, resulting in a short, incomplete translational product of approximately 50 aa in length that is degraded by proteases other than the proteasome (Fig. 7).

This model is also consistent with our conclusion that VIHCE occurs upstream of SEC61-mediated translocation because inhibition of translocation by ES₁ results in a full-length HC that is degraded by the proteasome. Eeyarestatin I and II were originally discovered in a screen for small molecule inhibitors of US11-mediated ER-associated degradation (ERAD) of MHC-I (14). This ERAD-stabilizing function correlates with the ability of ES₁ to bind to the AAATPase p97, a cytosolic chaperone that is essential for the extraction of misfolded proteins from the ER (57, 58). In addition, however, ES₁ was shown to inhibit protein translocation—both total protein secretion and that of model substrates (10). While it is not exactly known how ES₁ inhibits translocation, Cross et al. (10) concluded that ES₁ most likely directly targets the SEC61 complex. Similar to our finding with MHC-I HC, ES₁-inhibited substrates were shown to be degraded by the proteasome. Proteasomal degradation upon inhibition of protein translocation was also reported for other small molecule inhibitors of SEC61 translocation (cotransin/CAM471) that specifically target a subset of SP-containing proteins (4, 15). We therefore interpret the absence of a full-length HC in Rh178-containing cells treated with proteasome inhibitors as evidence that Rh178 prevents synthesis of full-length HC at a step prior to transfer to and/or translocation through the SEC61 translocon.

What, then, is the basis for Rh178 selectivity for the MHC-I HC? Structural studies of the SRP/SP complex suggest that, despite its hydrophobic nature, the SP is not completely buried within the SRP complex but is bound in a cleft-like structure, with some amino acids from the SP protruding from this cleft (24). Thus, we speculate that Rh178 might recognize specific HC-derived amino acids that protrude from the SRP complex, somewhat reminiscent of the T cell receptor recognizing peptide epitopes bound to the MHC-I groove. Alternatively, Rh178 may interfere with GTPase-mediated release of the SP from the SRP-SR complex, thereby blocking productive transfer of the RNC to SEC61.

In summary, this work further supports our previous conclusion that VIHCE represents a unique mechanism of viral interference with antigen presentation. Our results presented here suggest that Rh178 specifically interferes with SP-dependent HC translation at a point that precedes translocation but likely requires SRP-dependent SP recognition and transfer of the RNC to the ER membrane. Rh178 thus joins the growing number of viral proteins that are useful cell biology tools to dissect cellular pathways.

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