A Bipartite Trigger for Dislocation Directs the Proteasomal Degradation of an Endoplasmic Reticulum Membrane Glycoprotein

Polypeptides are organized into distinct substructures, termed protein domains, that are often associated with diverse functions. These modular units can act as binding sites, areas of post-translational modification, and sites of complex multimerization. The human cytomegalovirus US2 gene product is organized into discrete domains that together catalyze the proteasome-dependent degradation of class I major histocompatibility complex heavy chains. US2 co-opts the endogenous ER quality control pathway in order to dispose of class I. The US2 endoplasmic reticulum (ER)-lumenal region is the class I binding domain, whereas the carboxyl terminus can be referred to as the degradation domain. In the present study, we examined the role of the US2 transmembrane domain in virus-mediated class I degradation. Replacement of the US2 transmembrane domain with that of the CD4 glycoprotein completely blocked the ability of US2 to induce class I destruction. A more precise mutagenesis revealed that subregions of the US2 transmembrane domain differ in their ability to trigger class I degradation. Collectively, the data support a model in which US2-mediated class I degradation occurs as a highly regulated process where the US2 transmembrane domain and cytoplasmic tail work in concert to eliminate class I molecules. Host factors, including a signal peptidase complex, probably associate with the US2 molecule in a coordinated fashion to create a predislocation complex to promote the extraction of class I out of the ER. The results imply that the ER quality control machinery may recognize and eliminate misfolded proteins using a similar multistep regulated process.

Viruses deliver their genetic material into the host cell to initiate infection and begin the process of replication. Entry into the host cell can be accompanied by the activation and initiation of an immune response. As the pathogen replicates its genome, fragments of viral proteins may enter the cytoplasmic peptide pool and be loaded onto class I MHC molecules, heterotrimERIC complexes composed of a heavy chain, β2m, immunoglobulin light chain, and antigenic peptide. Indeed, the class I MHC antigen presentation pathway plays a major role in alerting the immune system to the presence of intracellular pathogens. Virus-specific CD8 + CTLs monitor class I MHC molecules at the cell surface for foreign peptides and aid in the elimination of infected cells. Considering that some viruses, particularly most DNA viruses, require extensive time periods to replicate their genetic material, it would be quite advantageous for the pathogen to evolve strategies to interfere with the presentation of viral peptides by class I MHC molecules.

The human cytomegalovirus (HCMV), the largest member of the Herpesviridae family, contains a double-stranded DNA genome of ~230 kb encoding for over 200 open reading frames. HCMV requires roughly 1–2 days in order to completely duplicate its genome, with replication occurring in a temporal fashion of immediate early, early, and late gene products, respectively. This abundance of genes encoded by HCMV could, theoretically, provide an arsenal of antigenic peptides for presentation by class I molecules to CTLs, thereby removing the infected cell and ending the viral life cycle prematurely. Clinically, CD8 + CTLs responding to peptides from several HCMV immediate early gene products have been isolated, whereas CTLs specific for more abundant early and late proteins remain to be found. HCMV has evolved several ingenious strategies by which it disarms this particular component of the immune response during the early phase of infection. The US (unique short) region of the HCMV genome encodes at least five ER-resident glycoproteins that modulate the cell surface expression of class I MHC molecules. The immediate early gene product US3 binds and limits the egress of peptide-loaded class I molecules from the ER, their site of synthesis. The US6 protein complexes with the transporter associated with antigen presentation within the ER lumen and prevents the conformational rearrangement of TAP necessary for peptide transport. US10 delays, but does not block, the transport of class I molecules within the ER. The early gene products US2 and US11 target newly synthesized class I molecules for dislocation from the ER, leading to the complete extraction of the heavy chain portion of the complex.

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1 A predoctoral trainee, supported by National Institutes of Health Grant AI060905-S1.

2 To whom correspondence should be addressed: Dept. of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1124, New York, NY 10029. Tel.: 212-241-5447; Fax: 212-241-7336; E-mail: Domenico.Tortorella@mssm.edu.

3 The abbreviations used are: MHC, major histocompatibility complex; aa, amino acid(s); BiP, immunoglobulin heavy chain-binding protein; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCMV, human cytomegalovirus; HLA, human leukocyte antigen; SPP, signal peptide peptidase.
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from the ER membrane and into the cytosol for destruction by the proteasome (11, 12). US2 and US11 have co-opted the cellular process known as ER quality control, which controls the proper assembly of nascent secretory proteins, in an effort to inhibit the cell surface presentation of class I MHC molecules (13, 14).

The HCMV US2 gene encodes for a 199-amino acid polypeptide that localizes to the ER as a type I membrane glycoprotein (15). Early studies of HCMV infection demonstrated that US2 was one of two loci within the viral genome that functioned to increase the turnover of class I MHC molecules (16). Expression of this gene product alone in human cells caused the rapid destabilization and dislocation of class I heavy chains out of the ER and into the cytosol for subsequent degradation by the proteasome (12). Therefore, expression of US2 in mammalian cells has been used as an invaluable tool to study the cellular processes of dislocation and degradation. Crystallographic studies of HCMV US2 established that the ER-lumenal domain of this protein contains an immunoglobulin-like fold that facilitates its association with class I MHC molecules and is required for subsequent dislocation of heavy chains (17, 18). US2 targets a population of class I molecules within a specific conformational state; US2 exhibits reactivity with only properly folded class I molecules (19). In addition, for the US2-mediated dislocation of class I heavy chains, US2 directs the modification of class I with anywhere from one to three molecules of ubiquitin prior to complete extraction from the ER (20). Signal peptide peptidase (SPP), an ER-resident protease involved in cleavage of signal peptides and processing of antigens for HLA-E molecules, binds to the US2 cytoplasmic tail and is important to catalyze US2-mediated class I degradation (21). Interestingly, the ER-resident chaperone BiP has also been implicated in US2- and US11-mediated degradation of class I (22). Although a complete model remains to be fleshed out, much of the data summarized here suggest a structured series of events beginning once US2 binds to a lumenal epitope on properly folded class I molecules. This US2-class I complex is then recruited to the dislocation channel by interaction with specific cellular proteins, thus allowing the dislocation substrate to be ubiquitinated as it is extracted from the ER membrane. Class I heavy chains are then directed to the proteasome for destruction.

Structural analysis of US2 predicts that this viral polypeptide is a multidomain protein with its class I binding domain at the NH2 terminus and its degradation domain at the COOH terminus. A US2 truncation mutant in which the COOH-terminal cytoplasmic tail has been deleted no longer directs the degradation of class I MHC heavy chains (23, 24). A more precise mutagenesis study showed that every third residue of the cytoplasmic tail was critical for US2-mediated class I degradation, offering up more insight into how US2 contacts the dislocation machinery (25). In this study, we sought to discern the function of the US2 transmembrane domain and its role in class I dislocation. By generating chimeric US2 constructs in which the transmembrane domain alone or both cytoplasmic tail and transmembrane domain were replaced with that of the human CD4 protein, we found that neither construct was competent to degrade class I heavy chains. In addition, a panel of substitution mutants in which increasingly smaller sections of the US2 transmembrane domain were swapped out for CD4 sequences demonstrated that specific regions of the transmembrane domain were more important in mediating heavy chain dislocation. We hypothesize that the US2 transmembrane domain, in conjunction with the cytoplasmic tail, recruits cellular factors to sites of dislocation in order to extract class I heavy chains from the ER membrane. It would be intriguing to attribute this ordered activation of cellular proteins, or “trigger effect,” to the cellular quality control machinery as a means to regulate the dislocation of misfolded proteins from the ER.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Human U373-MG astrocytoma cells and U373 transfectants that stably express US2 mutant constructs (see below) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal bovine serum, 1 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C (95% air, 5% CO2). Gp2–293 cells (BD Biosciences) were utilized to generate retroviruses (see below) and were cultured in medium similar to U373 cells. Rabbit polyclonal anti-US2 antibody and anti-class I heavy chain antibody were raised against the bacterially expressed lumenal domain of US2 (aa 15–140) and class I heavy chain (HLA-A2 allele, aa 25–366), respectively. The monoclonal antibody W6/32 recognizes properly folded class I molecules (26) and was purified from hybirdoma-cultured supernatant. Rabbit polyclonal antibody to signal peptide peptidase was purchased from Abcam. Antiglieraldehyde-3-phosphate dehydrogenase was purchased from Upstate Biotechnology. Monoclonal antibody to BiP was purchased from BD Transduction Laboratories.

cDNA Constructs—The US2-CD4 chimeric cDNA constructs (US2CD4 and US2CD4US2) were generated from the ligation of two PCR fragments, with one fragment containing sequences corresponding to either the CD4 transmembrane domain alone or the CD4 transmembrane domain and cytoplasmic tail. The US2 transmembrane domain amino acid cDNA constructs (US2/CD426/US2, US2/CD427/US2, US2/CD428/US2, and US2/CD429/US2) were amplified with a primer corresponding to the 5′ end of the US2 cDNA and a 3′ primer containing the appropriate CD4 sequences plus the amino acid isoleucine. A similar PCR/ligation approach was used to construct US2 transmembrane domain mutants 2–7 (US2CD4US2-2 through -7), in which differing fragments of the CD4 transmembrane domain were introduced into a backbone of the US2 lumenal domain and cytoplasmic tail. All of the constructs were initially cloned into pcDNA3.1(+) and then subsequently subcloned into the retrovirus vector pLgPW (25).

Retrovirus Transduction—Retrovirus vectors containing the respective US2 mutant cDNA constructs and the vesicular stomatitis virus-G envelope were transiently transfected simultaneously into gp2–293 cells (BD Biosciences) by a lipid-based protocol to produce a pseudotyped Moloney murine leukemia virus amphotropic retrovirus. In brief, pLgPW-US2 constructs and pSVS-G envelope protein (BD Biosciences) were transfected into gp2–293 cells on day 1, with the transfection media being changed every 24 h for 2 days. Seventy-two hours post-transfection, medium containing retrovirus particles was added to U373 cells with a final concentration of 8 μg/ml hexad-
imethrine bromide (Sigma). Enhanced green fluorescent protein-expressing cells were sorted using a MoFlo high speed cell sorter (Cytomation) (Mount Sinai Flow Cytometry Facility).

**Cell Lysis and Immunoprecipitation**—Cells (1.0 × 10⁶) were lysed in Nonidet P-40 lysis mix (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM MgCl₂, 5 mM EDTA, 0.5% (v/v) Nonidet P-40) supplemented with 1.5 μg/ml aprotinin, 1 μM leupeptin, and 200 μM phenylmethylsulfonyl fluoride) followed by the removal of nuclei and insoluble material using centrifugation (2 min, 16,100 × g at 4 °C). The lysates were incubated with the respective antibody followed by the addition of Pansorbin cells (Calbiochem) for 40 min on a nutator at 4 °C. The Pansorbin cells were pelleted and washed twice with wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40). The polypeptides were released from the Pansorbin cells with 1× SDS sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol blue, 50 mM dithiothreitol), resolved using SDS-PAGE (12.5% or 15%), and subjected to immunoblot analysis.

**Flow Cytometry Analysis**—Quantitative flow cytometry analysis of surface-expressed class I MHC molecules was assessed using W6/32 or HLA-A2 (1.5 μg of antibody/1 × 10⁶ cells), followed by incubation with Alexa 647-conjugated anti-mouse IgG (Molecular Probes). The fluorescence signal was measured from 10,000 cells using a Cytomics FC 500 flow cytometer (Beckman Coulter). The data were analyzed using FlowJo software (Tree Star, Inc.). The levels of surface class I molecules are represented by plots of normalized cell number versus fluorescence signal. Normalized cell number was calculated by setting the maximum cell number at the respective peak fluorescence value to 100.

**Immunofluorescence**—U373 cells and US2 transfectants were plated onto 13-mm coverslips in a 24-well plate at a concentration of 0.5 × 10⁶ cells/well. Approximately 12 h later, cells were washed twice in PBS at room temperature, fixed with methanol/acetic acid (1:1) for 10 min at 4 °C, washed twice with PBS, and then blocked for 30 min at room temperature in PBG (phosphate-buffered saline solution containing 0.5% bovine serum albumin, 0.2% (v/v) fish gelatin) supplemented with 0.075% saponin and 4% normal goat serum. Cells were incubated for 1 h at room temperature with both anti-US2 and anti-BiP antibody (1:500 each in PBG plus 0.075% saponin). Cells were washed four times with PBG and then incubated for 1 h at room temperature, in the dark, with both fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes) and Texas Red-conjugated goat anti-mouse antibody (Molecular Probes) diluted in PBG/saponin. Cells were washed four times with PBG, once with PBS, and once with distilled H₂O. The samples were then mounted on a glass side using ProLong Antifade solution (Invitrogen). Images were captured using an Olympus 1X70 fluorescence microscope and analyzed using QCapture Pro software (Media Cybernetics). Images were generated using Adobe Photoshop 7.0 (Adobe Systems, Inc.).

**RESULTS**

**HCMV US2 Transmembrane Domain Chimeras Are ER-localized Species**—US2 is one of two HCMV-encoded ER-resident proteins, the other being US11, that exploit the process known as ER quality control to target class I MHC molecules for degradation (16). HCMV US2 is a 199-amino acid type I membrane glycoprotein composed of an ER-lumenal portion (aa 1–160), transmembrane domain (aa 161–185), and a short cytoplasmic tail (aa 186–199). The ER-lumenal domain of HCMV US2 binds class I MHC molecules (17, 18), whereas the cytoplasmic tail is necessary to promote proteasomal degradation of heavy chain molecules (23–25). US2 and the homologous HCMV glycoprotein US11 have nonclassical transmembrane domains containing several polar or charged residues that are not usually found in transmembrane regions. These polar amino acids within the lipid bilayer have the capacity to facilitate the formation of protein complexes and mediate cellular processes (27). In fact, a glutamine residue at position 192 within the transmembrane domain of HCMV US11 is crucial for catalyzing degradation of class I heavy chains (28). Therefore, to ascertain the role of the US2 transmembrane domain (residues 161–185) in class I degradation, US2 chimeric mutants were generated in which either the US2 transmembrane domain alone was exchanged for CD4 sequences (US2CD4US2) or both the transmembrane domain and cytoplasmic tail of US2 were replaced with CD4 sequences (US2CD4) (Fig. 1A). The human CD4 is a type I membrane protein with a classical transmembrane domain containing only nonpolar residues. These chimeric US2 molecules were stably transduced into U373 cells, and expression of these US2 mutants was examined by an immunoprecipitation/immunoblot experiment (Fig. 1B). Total cell lysates and anti-US2 precipitates from equal numbers of U373 cells and US2 chimeric-expressing cells were subjected to SDS-PAGE and analyzed by an anti-US2 (Fig. 1B, lanes 1–8) or anti-GAPDH immunoblot (Fig. 1B, lanes 9–16). The anti-GAPDH immunoblot confirmed an equal loading of samples. US2 polypeptides were detected from the anti-US2 precipitates as well as from the total cell lysates (Fig. 1B, lanes 2–4 and 6–8). The US2CD4 Protein migrated more slowly than wild type US2199 as predicted by its molecular mass of ~24.8 kDa (Fig. 1B, lane 2 versus lane 3). Interestingly, US2CD4US2 also migrated more slowly than US2199, despite the fact that both molecules have an equal number of residues (Fig. 1B, lane 2 versus lane 4). In addition, two distinct polypeptides of US2CD4US2 were recovered with anti-US2 sera (Fig. 1B, lane 4), perhaps corresponding to a post-translational modification, such as the attachment of a single N-linked glycan. In total, these results demonstrate that the US2 transmembrane domain chimeric constructs were expressed in U373 cells.

To investigate whether the US2 chimeric mutants were processed as wild type US2 with regard to the acquisition of an N-linked glycan, we examined the sensitivity of each mutant to endoglycosidase H, an enzyme that removes high mannose N-linked glycans. US2 polypeptides recovered from the respective US2 chimeric cell line with anti-US2 sera were subjected to endoglycosidase H treatment. The precipitates were resolved on SDS-PAGE and analyzed by an anti-US2 immunoblot (Fig. 1C). As expected, wild type US2 (US2199) and US2CD4 were susceptible to endoglycosidase H cleavage (Fig. 1C, lanes 3 and 4 and lanes 7 and 8), as demonstrated by the faster migrating US2 species (Fig. 1C, lanes 3 and 8). The two polypeptides of US2CD4US2 (Fig. 1C, lane 5) were converted to one species...
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Expression and characterization of HCMV US2 transmembrane domain chimeric constructs. A, wild type US2_{199} is a type I membrane protein consisting of an ER-lumenal domain (aa 1–160) with a single glycosylation site (N68) (solid circles), a transmembrane domain (black box) (aa 161–185), and a short cytoplasmic tail (solid line) (aa 186–199) of 14 residues. The US2_{CD4} chimaera consists of the CD4 transmembrane domain (hatched box) and cytoplasmic tail (dashed line) (aa 395–458) in place of the respective US2 sequences. The US2_{CD4US2} chimaera consists of the US2 lumenal domain, the CD4 transmembrane domain (aa 395–419), and the US2 cytoplasmic tail. B, total cell lysates (lanes 5–8 and 13–16) and US2 precipitates from U373, US2_{199}, US2_{CD4}, and US2_{CD4US2} cells using an anti-US2 serum (lanes 1–4) were resolved on an SDS-polyacrylamide gel (15%) and subjected to immunoblot analysis using anti-US2 and anti-GAPDH antibodies. C, US2 precipitates from U373, US2_{199}, US2_{CD4}, and US2_{CD4US2} cells using anti-US2 serum were digested with or without endoglycosidase H (1 h, 37 °C) (EndoH). Samples were resolved on an SDS-polyacrylamide gel (15%) and subjected to immunoblot analysis using anti-US2 and anti-GAPDH antibodies. D, US2 precipitates from U373, US2_{199}, US2_{CD4}, and US2_{CD4US2} cells plated onto coverslips were treated with anti-US2 polyclonal serum and anti-BiP monoclonal antibody, followed by incubation with a goat-anti-rabbit immunoglobulin (FITC) and a goat-anti-mouse immunoglobulin (Texas Red). A merged image of both stains is seen in the right-hand column. For B and C, US2 proteins, GAPDH, and molecular standards are indicated.

Upon treatment with endoglycosidase H (Fig. 1C, lane 6). These results demonstrated that the faster migrating US2_{CD4US2} polypeptide was indeed a nonglycosylated form of US2_{CD4US2}. This result intriguingly implies the involvement of the US2 transmembrane domain in processing of the molecule. In total, the glycosylation patterns for each US2 transmembrane domain chimera mimic those of wild type US2_{199}. To further verify that the US2 chimeras reside within the ER, the cellular localization of the US2 proteins was examined using immunofluorescence microscopy. U373, US2_{199}, US2_{CD4}, and US2_{CD4US2} cells were plated onto coverslips and probed with both anti-US2 antibody and anti-BiP antibody, followed by incubation with a fluorophore-conjugated secondary antibody. Cells expressing each US2 chimera displayed a subcellular staining pattern similar to wild type US2_{199} (Fig. 1D). Consistent with published data, each cell line demonstrated a diffuse cytoplasmic staining for US2 and BiP, typical of endoplasmic reticulum localization (29). Collectively, the data demonstrates that the US2-CD4 chimeras retained the localization and post-translational modifications observed in wild type US2_{199} cells.

US2-CD4 Chimeras Are Deficient in US2-mediated Dislocation of Class I Molecules—To determine if the US2-CD4 chimeras target class I molecules for proteasome-dependent degradation, levels of class I heavy chains were analyzed in the absence and presence of proteasome inhibitor. The inclusion of proteasome inhibitor causes the accumulation of the glycosylated and deglycosylated, dislocated forms of heavy chains. Total cell lysates from equal numbers of U373, US2_{199}, US2_{CD4}, and US2_{CD4US2} cells, untreated or treated with the proteasome inhibitor ZL3VS (2.5 μM, 16 h), were subjected to an anti-heavy
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US2 transmembrane chimeras fail to promote degradation of class I MHC molecules in a proteasome-dependent fashion. Total cell lysates from U373, US2199, US2CD4, and US2CD4US2 cells untreated or treated with the proteasome inhibitor ZL3VS (16 h, 2.5 μM) were resolved on an SDS-polyacrylamide gel (12.5%) and subjected to immunoblot analysis using anti-class I heavy chain serum (lanes 1–8) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (lanes 9–16). Glycosylated class I heavy chains (HC(CHO)), deglycosylated class I heavy chains (HC(-CHO)), GAPDH, and molecular weight standards are indicated. * nonspecific polypeptides.

The binding of US2 to class I molecules is specific for HLA-A2 allele (Fig. 3B). As with W6/32 analysis, a significant reduction of surface class I was observed in US2199 cells (Fig. 3B, top), and both US2CD4 and US2CD4US2 failed to down-regulate surface class I molecules (Fig. 3B, middle and bottom). Since there was no significant decrease of HLA-A2 molecules in US2CD4 cells, the decrease of class I using W6/32 was probably due to a delayed ER exit of other class I alleles, such as HLA-B and HLA-C. Taken together, the data demonstrated that replacement of the US2 transmembrane domain with a classical transmembrane domain resulted in an ablation in the ability of the US2 chimera to induce class I degradation.

US2-CD4 Chimeras Maintain Their Amino-terminal Class I Binding Domains—The binding of US2 to class I molecules is probably a prerequisite for class I-induced degradation. It is a possibility that the ability of US2CD4 and US2CD4US2 to bind class I was lost upon exchange of the transmembrane of US2 with that of CD4, resulting in the aforementioned phenotype. To address this matter, a US2/class I association experiment was performed (Fig. 4). Class I molecules and US2 polypeptides were recovered from equal numbers of US2199, US2CD4, and US2CD4US2 cells using W6/32 antibody (Fig. 4, top) and anti-US2 antibody (Fig. 4, bottom), respectively. The W6/32 precipitates were subjected to a sequential immunoblot using anti-class I heavy chain sera and subsequently followed by anti-US2 sera (Fig. 4, top). As expected, a minute amount of class I heavy chains was recovered from wild type US2199 cells (Fig. 4, compare lanes 1 and 2). In accordance with previous findings that the US2 chimeras do not target class I for degradation, equivalent levels of class I molecules were recovered from US2CD4 and
US2<sub>CD4</sub>US2 cells when compared with U373 cells (Fig. 4, compare lane 1 with lanes 3 and 4). The anti-US2 immunoblot of the W6/32 precipitates revealed that US2<sub>CD4</sub> and US2<sub>CD4</sub>US2 polypeptides co-precipitated with class I molecules, demonstrating that US2<sub>CD4</sub> and US2<sub>CD4</sub>US2 continue to interact with class I molecules. The small amount of class I recovered from US2<sub>199</sub> cells would not allow us to visualize co-precipitated US2 protein (Fig. 4, lane 2). Immunoprecipitation of US2 polypeptides demonstrated that the total amounts of US2<sub>199</sub>, US2<sub>CD4</sub>, and US2<sub>CD4</sub>US2 were equivalent (Fig. 4, lanes 5–8). Thus, it can be inferred that the inability of US2<sub>CD4</sub> and US2<sub>CD4</sub>US2 to degrade class I was not due to a defective class I binding domain.

Modulation of the Transmembrane Domain of US2<sub>CD4</sub>US2 Does Not Promote Class I Degradation—The US2 cytoplasmic tail is directly involved in class I degradation, and a US2 mutant that lacks the carboxyl-terminal 14 residues fails to induce class I destruction (23, 24). Published data postulate that the US2 cytoplasmic tail adopts a 3<sub>10</sub>-helical configuration, with four critical residues (cysteine 187, serine 190, tryptophan 193, and phenylalanine 196) arranged along the same surface of the tail to trigger class I dislocation (25). In comparison with a classical α-helix, in which 3.6 residues revolve around the helical axis, a 3<sub>10</sub>-helix contains 3 residues/turn (30). Since the cytoplasmic tail most likely needs to be in a specific orientation to interact with the degradation apparatus, the critical residues within the cytoplasmic tail of the US2<sub>CD4</sub>US2 molecule may no longer lie along the correct plane or be the correct distance from the membrane to initiate class I degradation. To address this possibility, we generated four transmembrane domain amino acid mutants in which the transmembrane domain was either extended or retracted, thereby rotating the critical face of the 3<sub>10</sub>-helix (Fig. 5A). The US2<sub>CD4</sub>US2 construct, which contains the 25-amino acid CD4 transmembrane domain, is denoted US2/CD4<sub>25</sub>/US2 (Fig. 5A). To generate mutants in which the transmembrane domain was extended, the hydrophobic amino acid isoleucine was added to the transmembrane domain of the US2/CD4<sub>25</sub>/US2 construct (Fig. 5A, US2/CD4<sub>26</sub>/US2 and US2/CD4<sub>27</sub>/US2); for mutants in which the transmembrane domain was retracted, the amino acid phenylalanine was deleted from the end of the transmembrane domain of the US2/CD4<sub>25</sub>/US2 construct (Fig. 5A, US2/CD4<sub>22</sub>/US2 and US2/CD4<sub>23</sub>/US2). The ability of these mutants to degrade class I molecules was examined by analyzing levels of class I (Fig. 5B). Class I molecules were recovered from equal numbers of U373, US2<sub>199</sub>, US2/CD4<sub>23</sub>/US2, US2/CD4<sub>24</sub>/US2, US2/CD4<sub>25</sub>/US2, US2/CD4<sub>26</sub>/US2, and US2/CD4<sub>27</sub>/US2 cells using W6/32 antibody. Precipitates were subjected to an immunoblot using anti-class I heavy chain serum (Fig. 5B). Consistent with previous
findings, only a minute amount of class I was recovered from wild type US2 cells when compared with control U373 cells (Fig. 5B, compare lanes 1 and 2). As expected, US2/CD4<sup>179</sup>/US2 does not target class I for degradation, as observed by an abundance of recovered class I molecules (Figs. 2–4 and 5B, lane 5). Also, the additional transmembrane domain mutants (US2/CD4<sup>421</sup>/US2, US2/CD4<sup>424</sup>/US2, US2/CD4<sup>426</sup>/US2, and US2/CD4<sup>427</sup>/US2) do not target class I molecules for destruction (Fig. 5B, lanes 3 and 4 and lanes 6 and 7). Class I/US2 association experiments verified the ability of each mutant to continue to bind heavy chains.<sup>4</sup>

To ensure that each mutant construct was properly expressed, levels of US2 protein were confirmed from equivalent numbers of cells using an anti-US2 serum (Fig. 6A), anti-GAPDH (Fig. 6B), and anti-US2 sera. Class I heavy chains, US2 proteins, and molecular standards were recovered by immunoprecipitation with anti-US2 sera (Fig. 6C). The precipitates were resolved on an SDS-polyacrylamide gel (12.5%) and subjected to immunoblot analysis using anti-class I heavy chain serum (Fig. 6D). As expected, US2<sub>199</sub> proteins from each cell line were recovered by immunoprecipitation with anti-US2 sera (Fig. 6C). Interestingly, all cell lines expressed their respective US2 protein in two forms (Fig. 6C, lanes 3 and 5–9). This result led us to conclude that the nonglycosylated form of US2 observed in other transmembrane domain mutants (Figs. 1, 4, 5, and 6) was not contributing to the inability of the mutants to degrade class I. Cell lines expressing mutants 4 and 5, which induce degradation of class I, also gen-

<sup>4</sup>V. M. Noriega and D. Tortorella, unpublished observations.

**FIGURE 4. US2 transmembrane domain chimeras continue to associate with class I MHC molecules.** Top, properly folded class I molecules were recovered from U373, US2<sub>199</sub>, US2<sub>CD4</sub>, and US2<sub>CD4-US2</sub> cells with W6/32 (lanes 1–4). Bottom, US2 proteins were immunoprecipitated from the same samples with anti-US2 serum (lanes 5–8) to demonstrate equivalent amounts of US2 proteins. The precipitates were resolved on an SDS-polyacrylamide gel (12.5%) and subjected to immunoblot analysis using anti-class I heavy chain and anti-US2 sera. Class I heavy chains, US2 proteins, and molecular standards are noted.

for the CD4 transmembrane domain did not disrupt the US2 ER-lumenal class I binding domain or the orientation of the US2 carboxyl-terminal degradation domain. The loss of class I degradation by these mutants was truly due to the lack of the US2 transmembrane domain. Therefore, the US2 transmembrane domain plays a critical role in class I degradation.

A **Hierarchy Exists among Distinct Regions within the US2 Transmembrane Domain**—To further delineate the role of the US2 transmembrane domain in class I degradation, a panel of mutants was created in which different portions of the CD4 transmembrane domain were incorporated into a backbone of the US2 molecule (Fig. 6A). US2<sub>CD4-US2</sub>, which contains the CD4 transmembrane domain and has lost the ability to degrade class I molecules (Figs. 2 and 3), is henceforth termed mutant 1 (US2<sub>CD4-US2-1</sub>). US2 transmembrane domain mutants 2 and 3 (US2<sub>CD4-US2-2</sub> and -3) have had the amino-terminal end of the US2 transmembrane domain (aa 161–164 and 161–174) replaced with the CD4 transmembrane domain, whereas mutants 4 and 5 (US2<sub>CD4-US2-4</sub> and -5) have had the carboxyl-terminal end of the US2 transmembrane domain (aa 166–185 and 176–185) replaced with CD4 transmembrane sequences. Mutant 6 (US2<sub>CD4-US2-6</sub>) is composed of the amino-terminal 5 residues and the carboxyl-terminal 6 residues of the US2 transmembrane domain (aa 161–165 and 179–185), with the remaining internal sequences being those of CD4 (aa 400–412). Mutant 7 (US2<sub>CD4-US2-7</sub>) is composed of the first five residues and the last 10 residues of the CD4 transmembrane domain (aa 395–399 and 410–419).

Total cell lysates of U373 cells and cells that stably express the US2 transmembrane domain mutants (mutants 1–7) were subjected to immunoblot analysis with anti-class I heavy chain serum (Fig. 6B, lanes 1–9). Similar levels of class I were observed in cells expressing mutants 1, 6, and 7, and 7 when compared with U373 cells (Fig. 6B, compare lanes 3, 8, and 9 with lane 1). Mutants 2, 4, and 5 targeted class I for degradation, albeit at a lesser extent than US2<sub>199</sub> cells (Fig. 6B, lanes 4, 6, and 7). Mutant 3 appears to down-regulate class I to levels somewhere between US2<sub>199</sub> and U373 cells (Fig. 6B, lane 7). The results suggest that the entire US2 transmembrane domain is directly involved in class I degradation, since no mutant construct had the ability to degrade class I at levels seen in wild type US2. However, it appears that the amino terminus of the US2 transmembrane domain (as seen in mutant 4) may play a more influential role in class I degradation, whereas carboxyl-terminal residues (as seen in mutant 3) are less critical for US2 function. Flow cytometry experiments confirmed that mutants 2–5 down-regulated surface class I molecules to different levels (supplemental Fig. 1). Collectively, the data also imply that multiple regions within the transmembrane domain of US2 function together to efficiently target class I for destruction.

US2 proteins from each cell line were recovered by immunoprecipitation with anti-US2 sera (Fig. 6C). Interestingly, almost all cell lines expressed their respective US2 protein in two forms (Fig. 6C, lanes 3 and 5–9). This result led us to conclude that the nonglycosylated form of US2 observed in other transmembrane domain mutants (Figs. 1, 4, 5, and 6) was not contributing to the inability of the mutants to degrade class I. Cell lines expressing mutants 4 and 5, which induce degradation of class I, also gen-
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Small adjustments to the US2CD4 US2 transmembrane domain do not restore US2 degradation abilities. A, wild type US2 and US2 transmembrane domain amino acid mutants US2/CD4 (US2CD4) US2, US2/CD4 (US2CD4) US2, US2/CD4 (US2CD4) US2, and US2/CD4 (US2CD4) US2 are depicted in a schematic diagram. Single-letter amino acid codes are used to identify the respective residues. The total number of residues in the transmembrane domain is indicated for each mutant. B, properly folded class I molecules recovered from U373, US2CD4 US2, US2/CD4 US2, US2/CD4 US2, US2/CD4 US2, US2/CD4 US2, and US2/CD4 US2 cells with W6/32 were resolved on an SDS-polyacrylamide gel (12.5%) and subjected to immunoblot analysis using anti-class I heavy chain serum. C, total cell lysates from U373, US2CD4 US2, US2/CD4 US2, US2/CD4 US2, US2/CD4 US2, US2/CD4 US2, and US2/CD4 US2 were resolved on an SDS-polyacrylamide gel (15%) and immunoblotted using anti-US2 serum (lanes 1–7) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (lanes 8–14). For B and C, class I heavy chains, US2 proteins, GAPDH, and molecular standards are indicated.
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Entire US2 transmembrane domain removed (Fig. 4). Alternatively, the stability of the US2-class I complex may require an association with additional cellular proteins (possible signal for degradation revealed that mutants 1, 3, 6, and 7 also associated with SPP with different affinities. Mutants 1 and 3 strongly interacted with SPP when compared with mutants 6 and 7 (Fig. 8, lanes 7–9).

The US2-SPP complex is an important step in the induction of US2-mediated class I heavy chain degradation (21). US2 transmembrane domain mutant 1 cannot induce class I destruction, yet it continues to bind SPP (21), further corroborating the possibility that US2, more specifically the US2 transmembrane domain, is essential for mediating class I degradation. To examine whether the transmembrane domain mutants that cannot target class I for degradation continue to interact with SPP, we performed an SPP/US2 association experiment (Fig. 8). SPP molecules were recovered from U373, US2, and mutant 1–7 cells using anti-SPP antibodies. The specificity of the SPP antibody was demonstrated using U373 and US2199 cells (supplemental Fig. 2). Samples were resolved on SDS-PAGE and subjected to an anti-US2 immunoblot (Fig. 8, lanes 1–9) and anti-SPP (Fig. 8, lanes 10–18) immunoblot. Mutants 1, 3, and 4 complexed strongly with SPP (Fig. 8, lanes 3, 5, and 6), whereas mutants 2, 5, 6, and 7 associated with less affinity to SPP (Fig. 8, lanes 4 and 7–9). In these experiments, we also observed slower migrating polypeptides of mutants 1, 3, and 4 (Fig. 8, lanes 3, 5, and 6, asterisks). These molecules are most likely post-translationally modified versions of these mutants that occur upon prolonged association with SPP. Interestingly, there is no direct correlation between US2 binding to SPP and class I degradation. Mutants 2, 4, and 5 can induce class I degradation at similar levels yet interact with SPP at varying degrees. These results suggest that these US2 molecules form a transient complex with SPP prior to class I dislocation.

Examination of the US2 mutants that fail to target class I molecules for degradation revealed that mutants 1, 3, 6, and 7 also associated with SPP with different affinities. Mutants 1 and 3 strongly interacted with SPP when compared with mutants 6 and 7 (Fig.
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FIGURE 7. US2CD4US2 transmembrane domain mutants 1–7 continue to associate with class I molecules. Properly folded class I molecules were recovered using W6/32 from U373 cells, US2199 cells, and cells expressing US2 transmembrane domain mutants 1–7 (US2CD4US2-1 through US2CD4US2-7). The cells were treated with the proteasome inhibitor ZL3VS (2.5 μM, 16 h). Precipitates were resolved on an SDS-polyacrylamide gel (15%) and subjected to immunoblot analysis using anti-class I heavy chain (lanes 1–9) and anti-US2 sera (lanes 10–18). Class I heavy chains, US2 proteins, and molecular standards are indicated. *, nonspecific polypeptides.

FIGURE 8. US2CD4US2 transmembrane domain mutants 1–7 complex with signal peptide peptidase. U373, US2199, and mutants US2CD4US2-1 through US2CD4US2-7 cells treated with the proteasome inhibitor ZL3VS (2.5 μM, 16 h) were subjected to immunoprecipitation using anti-signal peptide peptidase sera. Samples were resolved on SDS-polyacrylamide gel (12.5%) and subjected to immunoblot analysis using anti-US2 (lanes 1–9) and anti-SPP (lanes 10–18) sera. US2 proteins, SPP, immunoglobulin heavy chain (lg HC), and molecular standards are indicated. *, slower migrating US2 polypeptides.

TABLE 1

| US2 structures | Class I MHC down-regulation |
|----------------|----------------------------|
| US2199         | ++                         |
| US2199         | --                         |
| US2CD4US2-1    | ++                         |
| US2CD4US2-2    | +                          |
| US2CD4US2-3    | +                          |
| US2CD4US2-4    | +                          |
| US2CD4US2-5    | +                          |
| US2CD4US2-6    | --                         |
| US2CD4US2-7    | --                         |

8, compare lanes 3 and 5 with lanes 8 and 9). This variation in SPP binding may be attributed to an altered conformation of the mutant’s cytoplasmic tail. An alternative hypothesis would propose that these mutants interact with the components of the predislocation complex and are unavailable to bind to SPP or class I. Collectively, these results imply that US2-mediated class I destruction is a multistep process that involves a complex interplay between class I molecules and cellular proteins, such as SPP.

DISCUSSION

The human cytomegalovirus US2 protein has co-opted the process known as ER quality control in an effort to circumvent the immune response triggered by viral infection (31). During the events of ER quality control, misfolded proteins are retained and ultimately disposed of before they can exit the ER. Although the class I molecule is competent to egress from the ER, US2 is able to bind class I, triggering a series of events leading to the ultimate dislocation of class I out of the ER followed by its degradation by the proteasome. The NH2-terminal domain of US2 forms a tight interaction with the class I molecule, but this binding step alone is insufficient to allow dislocation (17, 18). The COOH-terminal degradation domain is necessary to recruit the dislocation machinery and trigger the export of class I heavy chains into the cytoplasm (25). The data presented here demonstrate that the transmembrane domain of US2 is critical for mediating class I degradation. A mutant in which the transmembrane domain of US2 was replaced by the CD4 transmembrane domain (US2CD4US2) was incompetent to induce class I degradation (Fig. 2). The results demonstrate that the transmembrane domain of US2 must play an active role in US2-mediated class I dislocation.

The importance of the US2 transmembrane domain to trigger class I dislocation was further supported by data from the chimeric US2-CD4 mutants in which portions of the CD4 transmembrane domain were interspersed within US2 sequences (Fig. 6A). The US2 transmembrane domain was divided into three portions, based upon its unique composition (Fig. 6A); the nonclassical transmembrane domain of US2 contains 8 hydrophilic, polar amino acids (histidine, threonine, tyrosine, serine, and asparagine), unusual for a protein anchored within the lipid membrane of the ER (15). In fact, charged residues within a transmembrane domain can function as sites of protein complex assembly (32–36). Both histidine and serine are polar amino acids often found at the catalytic core of enzymes. Most cell signaling processes make use of serine, tyrosine, and threonine residues as sites for posttranslational modification (37). Therefore, these residues most likely play a regulatory role in US2-mediated class I degradation, either in transmission of a signal or in binding of cellular factors to the transmembrane domain.

The mutagenesis of the US2 transmembrane domain demonstrates the importance of differing portions of this domain and their necessity for US2-mediated class I destruction (Table 1). One specific region of the transmembrane domain that lies within the amino-terminal 5 residues can alone induce class I destruction. US2 transmembrane domain mutant 4 (US2CD4US2-4) efficiently down-regulated class I molecules, suggesting that these amino-terminal residues can induce the dislocation apparatus to promote class I extraction.

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Interestingly, the mutant 2 chimera (US2_{CD4}US2-2) that lacks the US2 NH_{2}-terminal 5 residues of the transmembrane domain continues to target class I for degradation. The mutant 2 chimera contains the carboxyl-terminal two-thirds of US2 transmembrane sequences. Mutant 5 (US2_{CD4}US2-5), which contains a combination of transmembrane segments seen in both mutants 2 and 4, was capable of degrading class I heavy chains, albeit not at levels seen in wild type US2 cells (Fig. 6B). The portion of the wild type US2 transmembrane domain conserved between these three mutants contains the aforementioned histidine, threonine, serine, and tyrosine residues (aa 161, 165, 169, and 170, respectively). These charged residues could theoretically create sites for docking of cellular factors or mediate signal transmission and promote the formation of a “dislocation competent” complex to extract class I heavy chains. Mutant 3 (US2_{CD4}US2-3) contains only the carboxyl-terminal 11 amino acids of the US2 transmembrane domain and had intermediate degradation capabilities (Fig. 6B). Interestingly, mutant 6 (US2_{CD4}US2-6), which contains the critical first 5 residues of the US2 transmembrane domain but also contains the residues seen in mutant 3, does not degrade class I. This leads to the hypothesis that perhaps the carboxyl-terminal end of the transmembrane domain may have a regulatory effect on US2-mediated dislocation. The carboxyl-terminal portion may limit the formation of a “dislocation-competent” complex. However, when the central portion of the transmembrane domain is expressed along with the COOH-terminal portion (mutant 2), dislocation capabilities are intact. It is possible that the central portion of the transmembrane domain counteracts the carboxyl-terminal regulatory effect and drives initiation of the dislocation reaction. Nonetheless, expression of central residues alone is not enough to drive dislocation, as evidenced by transmembrane domain mutant 7 (Fig. 6B).

What is the precise role of the US2 transmembrane domain in catalyzing class I degradation? The transmembrane domain may be involved in transmitting a signal from the ER lumen to the cytosol, in recruitment of cellular factors that mediate class I dislocation, or in a combination of both. We favor the paradigm that the transmembrane domain interacts with cellular proteins that induce extraction of class I, because the binding of SPP alone is not sufficient for class I destruction (Figs. 6 and 8) (21). In addition, US11-mediated class I degradation is mediated by the interaction through its transmembrane domain to derlin1 (38), and US2-induced class I degradation occurs using a similar mechanism. The engagement of these cellular proteins with the transmembrane can take place at different steps leading up to class I dislocation.

We propose several paradigms for class I dislocation. The common theme in these models is that dislocation occurs in a series of events involving the interaction of US2 with various cellular components (Fig. 9). One possibility is that the interaction between class I and US2 results in the recruitment of cellular proteins X, Y, and Z to the transmembrane domain and SPP to the cytoplasmic tail. This complex, in turn, may associate with the recruitment of the dislocation machinery and the ultimate disposal of class I heavy chains into the cytoplasm. In a second possible pathway, US2 engages cellular factors, such as SPP, prior to binding of class I. The US2 carboxyl terminus recruits these factors, but the US2 molecule is retained in a “dislocation-incompetent” form until it encounters a class I molecule. Binding of class I results in the transmission of a signal for dislocation from the ER-lumenal domain to the degradation domains of US2, and class I is destroyed. Interestingly, association experiments performed with the various transmembrane domain mutants provide data to support both models (Figs. 7 and 8). US2 transmembrane domain mutants 1, 3, 6, and 7, which fail to degrade class I molecules, provide us with perhaps the most information. Mutant 1 (US2_{CD4}US2-1) binds both class I and SPP very well yet is incapable of degrading class I. This mutant would fall in line with our first proposed model, where its continued ability to interact with class I results in a recruitment of SPP to the cytoplasmic tail. However, the
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CD4 sequences of mutant 1 do not allow this chimera to recruit the necessary factors to the transmembrane domain to initiate dislocation. In contrast, mutant 3 (US2<sub>CD4</sub>US2-3) binds SPP efficiently, yet it binds class I less strongly. Mutant 3 suggests, as our second model proposes, that US2 binds cellular proteins prior to class I. Because mutant 3 cannot recruit necessary transmembrane domain factors, it is deficient in class I binding and degradation. Mutants 6 and 7 (US2<sub>CD4</sub>US2-6 and -7) bind quite poorly to SPP and are impaired in class I binding. This would corroborate the idea that cellular factors bind the US2 transmembrane domain and cytoplasmic tail first. Therefore, if mutants 6 and 7 cannot bind SPP strongly, then they will be unable to associate well with class I. There is also the possibility that, for mutants that do not degrade, the loss of US2 sequences hinders the transmission of a signal from the ER-lumenal domain to the cytoplasmic tail or simply does not allow for the docking of necessary cellular factors to the transmembrane domain.

We favor a paradigm that SPP binds the US2 cytoplasmic tail in a predislocation step, retaining the degradation domain of US2 in a relaxed state. The ER-lumenal domain of US2 then interacts with a class I molecule. In a manner similar to signal transduction across biological membranes (39), the transmembrane domain of US2 relays the message for dislocation to the cytoplasmic tail through recruitment of cellular proteins X, Y, and Z. This recruitment most probably occurs through the aforementioned hydrophilic residues of the transmembrane domain. This US2-class I-cellular factor complex triggers the 14-residue carboxyl-terminal tail of US2 to undergo a conformational change in a Ta<sub>helical</sub> structure (25). This very specific structure induces changes in the US2 polypeptide, resulting in contact with the as-yet-identified dislocation pore. Class I heavy chains are then extracted from the ER membrane and deposited in the cytosol for degradation by the proteasome (Fig. 9). Although we support this model, what is quite clear from the data is that the trigger for dislocation emerges from a concerted effort among the US2 transmembrane domain and cytoplasmic tail.

Many questions still remain regarding US2-mediated class I degradation, the most pertinent being what are the cellular factors that US2 interacts with to trigger dislocation? How do these cellular proteins regulate US2 and, in turn, the degradation of class I? The fact that US2-mediated class I degradation is highly regulated by cellular proteins has far reaching implications for the process of ER quality control. How does the ER quality control machinery discern between proteins that are in the process of folding and those that terminally misfolded? Perhaps the binding of a misfolded protein to a chaperone must be followed by a second trigger informing the ER quality control machinery that this particular protein is a substrate for dislocation. The identification of interacting partners for the ER quality control machinery already provides some clues as to the components that US2 may be cooperating with to promote class I destruction. Key candidate proteins include cellular ubiquitin ligases to modify class I for extraction and destruction as well as ER membrane factors that can diffuse along the plane of the membrane to engage the dislocation pore. The classification of the proteins involved in this multistep trigger could aid in the identification of possible targets for therapeutic intervention to HCMV disease.

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