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HUMAN CYTOMEGALOVIRUS US3 MODULATES DESTRUCTION OF MHC CLASS I MOLECULES

Vanessa M. Noriega^a, Julia Hesse^b, Thomas J. Gardner^a, Katrin Besold^{b,1}, Bodo Plachter^b, and Domenico Tortorella^{a,*}

^aMount Sinai School of Medicine, Department of Microbiology, New York, New York 10029

^bInstitute for Virology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

Abstract

Human cytomegalovirus (HCMV), a member of the *Herpesviridae* family, is proficient at establishing lifelong persistence within the host in part due to immune modulating genes that limit immune recognition. HCMV encodes at least five glycoproteins within its unique short (US) genomic region that interfere with MHC class I antigen presentation, thus hindering viral clearance by cytotoxic T lymphocytes (CTL). Specifically, US3 retains class I within the endoplasmic reticulum (ER), while US2 and US11 induce class I heavy chain destruction. A cooperative effect on class I down-regulation during stable expression of HCMV US2 and US3 has been established. To address the impact of US3 on US11-mediated MHC class I down-regulation, the fate of class I molecules was examined in US3/US11-expressing cells and virus infection studies. Co-expression of US3 and US11 resulted in a decrease of surface expression of class I molecules. However, the class I molecules in US3/US11 cells were mostly retained in the ER with an attenuated rate of proteasome destruction. Analysis of class I levels from virus-infected cells using HCMV variants either expressing US3 or US11 revealed efficient surface class I down-regulation upon expression of both viral proteins. Cells infected with both US3 and US11 expressing viruses demonstrate enhanced retention of MHC class I complexes within the ER. Collectively, the data suggests a paradigm where HCMV-induced surface class I down-regulation occurs by diverse mechanisms dependent on the expression of specific US genes. These results validate the commitment of HCMV to limiting the surface expression of class I levels during infection.

Keywords

Antigen presentation; HCMV US3 and US11; Proteasome degradation; Immune modulation; MHC class I molecules

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*Corresponding author: Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1124, New York, NY 10029. Tel: 212-241-5447; Fax: 212-241-7335; Domenico.Tortorella@mssm.edu.

¹Present address: Novartis Pharma AG, Werk Klybeck, Klybeckstrasse 141, CH-4057 Basel Switzerland, Katrin.Besold@novartis.com

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1. INTRODUCTION

Viruses are organisms that subvert the cellular machinery of the host in order to replicate their genetic material and create infectious progeny. This dependence on living hosts for reproduction creates a unique interplay between these invaders and the components of the immune system. Indeed, the earliest steps of viral entry into the cell can be accompanied by the activation and initiation of a potent immune response (Liu et al., 2011). The ability of the host to recognize viral infection becomes paramount to halting the progression of disease and containing dissemination. The remarkable human immune defenses are highly coordinated and rely on the interaction of secreted proteins, receptor-mediated signaling, and intimate cell-cell communication. The competence of the host immune response determines how quickly and efficiently the pathogen is resolved as well as the severity of infection.

The interplay between virus and host is dynamic and evolution has seemingly provided viruses with mechanisms for bypassing, modulating, or disarming many facets of host immune defenses (Powers et al., 2008). Indeed, the genomes of many successful pathogens encode gene products that modify numerous steps of the immune response (Tortorella et al., 2000b). Human cytomegalovirus (HCMV) has the ability to initiate productive, or lytic, replication or to undergo a latent phase of infection with periodic bouts of reactivation. In order to persist indefinitely within the host, HCMV has evolved elaborate strategies to subvert cellular immune responses (Jackson et al., 2010). Viral functions targeting antigen presentation by major histocompatibility complex (MHC) class I molecules have been well documented (Tortorella et al., 2000a). By attenuating signaling by MHC class I molecules the virus circumvents clearance for a period of time by pathogen-specific cytotoxic T-lymphocytes (CTLs), thus allowing replication at a time when the virus is most vulnerable to host responses.

The unique short (US) region of the HCMV genome encodes at least five endoplasmic reticulum (ER) resident glycoproteins (US2, US3, US6, US10, and US11) that modulate the cell surface expression of MHC class I molecules (Loenen et al., 2001). In addition, the major tegument protein pp71 has been suggested to modulate class I surface expression (Trgovcich et al., 2006). Each of these proteins is expressed at distinct phases of the viral life cycle and appear to target individual components of the MHC class I antigen presentation pathway. The immediate early US3 gene product retains class I molecules within the ER through inhibition of the tapasin/TAP interaction necessary for optimal peptide loading, in combination with modulating protein disulfide isomerase (PDI) stability (Park et al., 2004; Park et al., 2006). The US2 and US11 genes encode early viral proteins that cause the rapid destabilization and destruction of class I heavy chains through a process mimicking endogenous ER quality control (Wiertz et al., 1996a; Wiertz et al., 1996b). Infection with HCMV mutants expressing either US2 or US11 demonstrate an incomplete protection from CD8⁺ T-cell recognition (Besold et al., 2009), while co-expression of US2 and US3 resulted in almost complete down-regulation of MHC class I molecules from the cell surface (Noriega and Tortorella, 2009).

In this study, we examined the fate of class I molecules during the expression of HCMV US3 and US11. The co-expression of these viral proteins in cells led to enhanced ER-retention of class I and diminished proteasomal degradation properties, thus demonstrating a dominance of US3 function over US11. Furthermore, infection with viral mutants expressing both US3 and US11 corroborated these findings during the course of infection. This novel relationship between viral-encoded immune modulators demonstrates a more complex control of host defenses by HCMV than initially suspected.

2. MATERIALS AND METHODS

2.1. Cells

Human U373-MG astrocytoma cells, U373 transfectants that stably express HCMV US gene products, and MRC5 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum (FBS), 1 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37° C in a humidified atmosphere (95% air/ 5% CO₂). Gpg-293 cells (BD Biosciences) were utilized to generate retroviruses and were cultured in media similar to U373 cells. Primary human foreskin fibroblasts (HFF) were grown in minimal essential medium (MEM; PAA, Cölbe, Germany), supplemented with 5–10% FBS (Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine, 50 mg/L gentamycin and 0.5 ng/mL basic fibroblast growth factor (bFGF; Invitrogen, Karlsruhe, Germany).

2.2. Antibodies and cDNA constructs

Rabbit polyclonal anti-US11 antibody was raised against the bacterially expressed GST-tagged luminal domain of US11 (aa. 21–180). Rabbit polyclonal anti-US3 antibody was a gift from Dr. Hidde Ploegh (Whitehead Inst., Cambridge, MA). The monoclonal antibody W6/32, recognizing properly folded class I molecules, was purified from hybridoma cultured supernatant. Rabbit polyclonal antibody to class I heavy chain was raised against the bacterially expressed luminal domain of HLA-A2 allele (aa. 25–308). Anti-glyceraldehyde-3-phosphate dehydrogenase was purchased from Upstate Biotechnology. US3 cDNA was cloned into the vector pLpCX (Clontech) and stably introduced into U373 astrocytoma cells by retroviral transduction. US3-expressing cells were clonally expanded in the presence of puromycin (0.375 µg/mL). U373-US3 cells were then transduced with a US11 retrovirus (retroviral vector pMIg) expressing GFP. The cells were sorted for GFP signal, thus isolating cells that only express US3 and US11. A similar cloning strategy was used to generate US2/US3 cells (Noriega and Tortorella, 2009).

2.3. BAC mutagenesis

The generation of RV-KB6 and RV-KB9 has been described elsewhere (Besold et al., 2007; Besold et al., 2009). Viral mutant RV-KB7 was generated analogously by BAC mutagenesis of the HCMV BAC pAD/Cre (Yu et al., 2002), using Red recombination in *Escherichia coli* strain EL250 as described by Lee and colleagues (Lee et al., 2001). In this process, the open reading frames (ORF) US2, US6 and US11 were sequentially deleted from the HCMV genome. As a first step, ORF US2 was deleted by inserting a kanamycin resistance (Kan^R) gene. The Kan^R gene, flanked by FRT sites, was amplified from a derivative of vector pCP15 (Cherepanov and Wackernagel, 1995), using engineered primers. These primers contained, in addition to the priming sequence for the Kan^R FRT cassette amplification, at their very 5'-ends about 50 bp of homology to the nucleotide sequences directly adjacent to US2 (primer KB1: 5'-ATGGGTACTCGTGGCTAGATTTATTGAAATAAACCGCGATCCCGGGCGTCTCGA GAA ACGCAGCTTC-3', primer KB2: 5'-CTCTGGGATATAAATTGGGAAAGAGCGTACAGTCCACACGCTGTTTCACCGGTA CCC GGGGATCTTG-3'). The amplified Kan^R gene construct was inserted in the viral DNA by homologous recombination, thereby replacing ORF US2; individual colonies were selected by addition of kanamycin. To remove the Kan^R gene from the BAC, individual colonies were streaked out. FLP expression was induced by arabinose as originally described by Lee and colleagues (Lee et al., 2001). The FLP recombinase removed the Kan^R gene from the viral DNA by site-specific FLP recombination at flanking FRT sites. The same strategy was then used in the second step to remove US11 (primer KB7: 5'-GGTGAGTCGTTTCCGAGCGACTCGAGATGCACTCCGCTTCAGTCTATATAGGTA CCCGGGGATCTTG-3', primer KB8: 5'-

TTACAGCTTTTGAGTCTAGACAGGGTAACAGCCTTCCCTTGTAAGACAGATCGA GAA ACGCAGCTTC-3'). Again, the Kan^R gene was removed by Flp recombination. Finally, for deletion of ORF US6 in the third step, insertion of an ampicillin resistance (Amp^R) gene was used, which was also amplified from the derivative vector of pCP15 (primer KB5: 5'-GAGAATGCCGTGTTGAAGGAACGCGCTTTTATTGAGACGATAAAACAGCAGCG GAA CCCCTATTTGTT-3', primer KB6: 5'-GAACATATATAATCGCCGTTTCGTAAGCACGTCGATATCACTCCTTCACTCTTGG TCT GACAGTTACC-3'). To avoid insertion of another FRT site into the HCMV genome, the Amp^R gene construct lacked FRT sites. Therefore, one copy of the Amp^R gene is contained in the final BAC pKB7.

2.4. Viral infection

Reconstitution of the wild type strain RV-BADwt and viral mutants as well as the generation of viral stocks were performed as described (Besold et al., 2009). Virus stock titration was performed by counting IE1 positive cells 48 hours post infection, following staining with a monoclonal antibody (mAb) against IE1 (p63-27; (Andreoni et al., 1989)). Multiplicity of infection (m.o.i.) was defined as the number of IE1 positive cells. For cytofluorometric analyses HFF were infected at an m.o.i. of 5. For co-infection experiments, an m.o.i. of 5 was used for each virus.

2.5. Cell lysis, immunoprecipitation, and flow cytometry analysis

Cell lysis and immunoprecipitations were carried out as previously described (Noriega and Tortorella, 2009). Proteasome inhibitor (carboxylbenzyl-leucyl-leucyl-leucine vinyl sulfone [ZL₃VS]) was a kind gift from Dr. Matthew Bogoy (Stanford University, Stanford, CA) and Dr. Hidde Ploegh (Whitehead Institute, Cambridge, MA). Samples were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis. Quantitative flow cytometry analysis of surface MHC class I molecules was assessed as previously described (Noriega and Tortorella, 2008) using a Cytomics FC 500 flow cytometer (Beckman Coulter). Plots of surface class I levels are represented by normalized cell number versus fluorescence signal. Flow cytometry of infected HFF was performed as previously described (Besold et al., 2009).

2.6. Pulse-chase analysis

Pulse-chase experiments of U373 stable transfectants were performed as previously described (Noriega and Tortorella, 2009). For virus infection, MRC5 fibroblasts were harvested 12 hours post infection with AD169 viral mutants and pulsed with ³⁵S-methionine for 3 hours at 37°C. Cells were lysed in NP40 lysis mix and immunoprecipitated for W6/32, US11, US3, and PDI, followed by incubation with protein A agarose beads (RepliGen). Samples were treated with 1X SDS sample buffer and resolved using SDS-PAGE. Protein A agarose beads containing W6/32 precipitants were then incubated with 500 units of Endoglycosidase H_f (New England Biolabs) for 2 hours and resolved using SDS-PAGE.

2.7. Immunofluorescence

U373 cells and US transfectants were prepared as previously described (Noriega and Tortorella, 2008). Briefly, cells were fixed in methanol:acetone, then washed and permeabilized in 0.1% saponin. Samples were treated with the monoclonal antibody W6/32 followed by incubation with a goat-anti-mouse secondary conjugated to Texas Red (Molecular Probes). Images were visualized and captured using an Olympus 1X70 fluorescence microscope and analyzed using Q Capture Pro software (Media Cybernetics). Images were generated using Adobe Photoshop 7.0 (Adobe Systems, Inc.).

3. RESULTS

3.1. Surface class I expression is reduced in US3/US11-expressing cells

The expression of HCMV gene products occurs in a tightly regulated cascade of immediate early, early, and late phases of replication (Mocarski Jr, 2007). One of the first gene products to be visualized within the infected cell is the immune modulator US3, between 1 and 8 hours post infection (Ahn et al., 1996). This is followed closely by the appearance of US2 and US11 transcripts at approximately 6 hours after infection (Besold et al., 2009). These viral proteins are all present within the ER of infected cells during the transition from immediate early to early phases of the life cycle and function to limit the surface expression of newly synthesized MHC class I molecules. In fact, stable expression of both US2 and US3 results in the enhanced turnover of MHC class I heavy chains (Noriega and Tortorella, 2009). To define the combined effect US3 and US11 have on class I trafficking, class I was examined in cells that stably express US3, US11, and both US3 and US11 (Material and Methods) (Figure 1A). Protein expression was confirmed from total cell lysates of U373 (control), US3, US11, and US3/US11 cells (Figure 1A, lanes 1–8). An anti-GAPDH immunoblot confirmed equivalent protein loading (Figure 1A, lanes 9–12). These cells were utilized to study the impact of US3 on US11-mediated class I degradation.

We next examined the surface expression of class I molecules in US3/US11 cells using flow cytometry (Figure 1B). U373, US3, US11, and US3/US11 cells were incubated with W6/32 monoclonal antibody followed by incubation with an anti-mouse immunoglobulin conjugated to AlexaFluor 647 (Figure 1B). US3 cells expressed almost equivalent levels of cell surface class I as U373 control cells (Figure 1B, top panel, dashed line). Class I molecules retained within the ER by US3 eventually traffic to the cell surface (Noriega and Tortorella, 2009). Cells that express US11 demonstrated decreased amounts of surface class I (Figure 1B, center panel, dashed line). Remarkably, US3/US11 cells demonstrated a robust class I down-regulation (Figure 1B, bottom panel, dashed line). The uniform down regulation of class I implies that the cells all express the US11 gene product. The results suggest that the concomitant expression of both US3 and US11 induces the efficient down-regulation of surface MHC class I molecules.

3.2. Class I molecules in US3/US11 cells are not targeted for proteasome destruction

To investigate the fate of class I molecules in US3/US11 cells, total cell lysates and properly folded class I molecules recovered from U373, US3, US11 and US3/US11 cells were subjected to immunoblot analysis (Figure 2A). Abundant levels of class I molecules were observed from both control U373 and US3 cells (Figure 2A, lanes 1–2 and lanes 5–6). Class I levels were significantly reduced in US11 cells (Figure 2A, lanes 3 and 7), consistent with published data (Wiertz et al., 1996a). Unexpectedly, increased amounts of total class I molecules and properly folded class I were observed from cells expressing both US3 and US11 (Figure 2A, lanes 4 and 8). The recovery of folded class I molecules is indicative of class I escaping US11-mediated destruction, thus allowing proper folding of class I proteins. To further validate these results, class I molecules were rescued from degradation in US11-expressing cells transduced with US3 (Supplemental Figure 1). Although US3 enhanced class I down-regulation in US2-expressing cells (Noriega and Tortorella, 2009), the data suggests that US3 may prevent efficient US11-mediated heavy chain degradation.

We next examined the localization of the class I molecules in US3/US11 cells. To do so, we determined class I sensitivity to endoglycosidase H (EndoH) (Figure 2B). Endo H cleaves high-mannose N-linked glycans of ER resident glycoproteins (Wiertz et al., 1996a). Total cell lysates from U373, US3, US11, and US3/US11 cells were treated with EndoH and subjected to immunoblot analysis (Figure 2B). As expected, steady state levels of class I

heavy chains from U373 cells were resistant to EndoH cleavage, as these molecules have exited the ER and enter the secretory pathway (Figure 2B, lanes 1–2). EndoH treatment of US3 cells resulted in generation of both EndoH sensitive and resistant forms of class I heavy chains (Figure 2B, lanes 3–4) due to transient retention within the ER. As expected, little to no class I was recovered from US11 cells (Figure 2B, lanes 5–6). Strikingly, in US3/US11 cells, EndoH treatment demonstrated that nearly all of the class I molecules were sensitive to EndoH cleavage (Figure 2B, lane 7–8, HC(–)CHO). An anti-GAPDH immunoblot confirmed equivalent protein loading (Figure 2B, lanes 9–16). These results revealed that class I molecules in US3/US11 cells are mostly retained in the ER, thus validating the class I surface down-regulation observed in these cells. Collectively, the data demonstrated that the function of US3 to retain class I molecules becomes the dictating characteristic in US3/US11 cells.

Immunofluorescence microscopy was utilized to further validate the cellular localization patterns of class I molecules in US3/US11 cells (Figure 2C). U373, US3, US11, and US3/US11 cells were fixed and probed with W6/32 antibody followed by incubation with a fluorophore-conjugated secondary antibody. Control U373 cells demonstrated diffuse staining for class I molecules, evidence of the subcellular trafficking of class I through the secretory pathway and towards the cell surface (Figure 2C, top left). US3 cells demonstrated more punctuate staining, further confirming that class I molecules in US3 cells were sequestered within the ER (Figure 2C, top right). As expected, minute amounts of class I molecules were visualized in US11 cells (Figure 2C, bottom left). Surprisingly, staining of US3/US11 cells was most similar to class I observed in US3 cells (Figure 2C, bottom right). Class I molecules in US3/US11 cells appear to be retained within the punctate structures seen in US3 cells, most likely the endoplasmic reticulum. These results further demonstrate the uniformity of the cell population. Collectively, the data demonstrates that the ER-retention properties of US3 are the dominating characteristic of US3/US11 cells.

US11-mediated class I degradation is driven by the intramembrane interaction of class I and a critical glutamine residue within the US11 transmembrane domain at amino acid 192 (Lilley et al., 2003). A US11 transmembrane point mutant (US11_{Q192L}) is able to bind to class I molecules, yet unable to induce class I degradation likely due to its inability to interact with the dislocation/degradation machinery (Lilley and Ploegh, 2004; Lilley et al., 2003). Conceivably, US3 function dominates in the presence of US11 by limiting class I engagement with US11 and thus the degradation machinery. To test this possibility, we examined whether the US11_{Q192L} that can only interact with class I molecules could block the function of US3. Total cell lysates and W6/32 precipitates from U373, US3, US11_{Q192L}, and US3/US11_{Q192L} cells were subjected to immunoblot analysis (Supplemental Figure 2A). Increased amounts of class I were recovered from US3/US11_{Q192L} cells in comparison to US11_{Q192L} cells alone (Supplemental Figure 2A, compare lanes 3 and 4 and lanes 7 and 8) suggestive of enhanced ER retention. Further, class I molecules recovered from US3/US11_{Q192L} cells were completely sensitive to EndoH digestion as compared to US11_{Q192L} cells (Supplemental Figure 2B, lanes 5–6 and 7–8) indicating that class I is retained in the ER. Taken together, the data suggest that US3 has the ability to enhance ER retention likely acting upon class I prior to US11.

3.3. HCMV US3 attenuates US11-mediated class I destruction

Are MHC class I molecules completely excluded from US11-mediated degradation in the presence of US3? To address this question, the rate of class I heavy chain turnover was examined in US3/US11 cells by pulse-chase analysis (Figure 3A). U373, US3, US11, and US3/US11 cells were metabolically labeled with ³⁵S-methionine for 15 minutes and chased up to 30 minutes. The class I heavy chains (Figure 3A, lanes 1–12), properly folded class I molecules (Figure 3A, lanes 13–24), US11 proteins (Figure 3A, lanes 25–36), and US3

proteins (Figure 3A, lanes 37–48) were recovered from cell lysates and resolved on a polyacrylamide gel. As expected, class I heavy chains and folded class I molecules were recovered from U373 and US3 cell lysates throughout the chase periods (Figure 3A, lanes 1–6 and 13–18). There is usually a slight decrease in class I heavy chains toward the latter chase points due to their conversion to slower migrating processed forms of class I molecules (Figure 3A, asterisk). Little to no class I was recovered from US11 cells (Figure 4A, lanes 7–9 and 19–21) due to the rapid kinetics of heavy chain degradation. Consistent with published data (Lilley et al., 2003), a small amount of class I was matured in US11 cells (Figure 3A, lane 9). Interestingly, class I molecules from US3/US11 cells were stabilized over the course of the chase (Figure 3A, lanes 10–12 and 22–24) in comparison to US11-expressing cells. Expression of US3 and US11 was confirmed by sequential recovery of these viral proteins (Figure 3A, lanes 25–36 and 37–48). These results demonstrated that expression of US3 significantly attenuates US11-mediated class I heavy chain degradation.

We further examined the effects of US3 on US11-mediated class I dislocation by determining the levels of deglycosylated class I intermediates. Class I molecules recovered from U373, US3, US11, and US3/US11 cells untreated or treated with the proteasome inhibitor ZL₃VS were subjected to immunoblot analysis (Figure 3B). In the absence of proteasome inhibition, little to no class I molecules were recovered from US11 cells (Figure 3B, lane 5). The inhibition of proteasome function caused the accumulation of deglycosylated class I degradation intermediates in US11 cells (Figure 3B, lane 6). Consistent with previous data (Figure 2), class I molecules were observed from US3/US11 cells (Figure 3B, lane 7) and treatment with proteasome inhibitor caused the accumulation of glycosylated and deglycosylated class I polypeptides (Figure 3B, lane 8). The levels of deglycosylated class I mirrored the amount observed in US3-expressing cells (Figure 3B, lane 4). The levels of class I deglycosylated intermediates observed in US3/US11 cells suggest that US11-mediated dislocation may still occur, but at an increasingly reduced rate. The data implies that expression of US3 radically decelerates the dislocation and degradation kinetics of class I heavy chains by HCMV US11.

3.4. US3/US11– induced MHC class I down-regulation in HCMV infected cells

To characterize the function of US3 and US11 on class I molecules during a virus infection, surface class I molecules were examined in infected cells using recombinant HCMV AD169 mutant viruses expressing either US3 or US11 gene products (Figure 4, US3+ and US11+ (Besold et al., 2007; Besold et al., 2009)). A mutant virus lacking the gene products US2, US3, US6 and US11 (Δ US2-11) was used as a control for deficiency in class I down-regulation. Growth kinetics and expression of US3– and US11-expressing mutant virus were indistinguishable from the parental strain RV-BADwt (data not shown, (Besold et al., 2009)).

The total class I levels were analyzed in fibroblasts infected with wild type, US3+, US11+, or US3+ and US11+ AD169 viruses (moi=5) by flow cytometry (Figure 5A). The levels of total class I molecules decreased over the infection period in wild type AD169 infected cells (Figure 5A, filled circle). In contrast, levels of surface class I molecules in US3+ or US11+ virus infected cells only transiently decreased over a similar period, with peak down-regulation occurring at 48 hours post-infection (Figure 5A, open circle and filled square). Strikingly, a decrease in class I levels similar to wild type AD169 was observed in cells co-infected with both US3+ and US11+ viruses (Figure 5A, open square). These results were similar to increased down-regulation of surface class I molecules observed in US3/US11-expressing cells (Figure 1B). The data suggests that during HCMV infection, US3 and US11 are able to effectively down-regulate class I molecules.

To address whether class I molecules are retained in the ER upon expression of US3 and US11 during an infection, the EndoH sensitivity of newly synthesized class I molecules were examined from virus-infected cells (Figure 5B). Human fibroblasts mock infected or infected with wild type, US3+, US11+, US3+ and US11+, and Δ US2-US11 AD169 viruses (moi=5) were harvested at 12 hours post-infection and metabolically labeled with 35 S-methionine for 3 hours. US3/US11-expressing cells were included as a control (Figure 5B, lanes 1–2 (–)). Properly folded class I complexes were recovered using W6/32 and further subjected to EndoH digestion. Mock infected samples demonstrated steady state levels of class I molecules (Figure 5B, lanes 3–4). Furthermore, these class I molecules traffic normally to the cell surface and are resistant to EndoH digestion. In comparison, cells infected with wild type AD169 demonstrate reduced recovery of class I molecules, as this virus expresses all encoded immune evasion molecules (Figure 5B, lanes 5–6). The remaining class I molecules observed in wild type AD169 infected cells are likely due to a small population of uninfected cells in which the class I escapes the ER and acquires EndoH resistance. Class I levels are slightly decreased in US11+ virus infected cells and completely resistant to EndoH (Figure 5B, lanes 11–12), while US3+ virus infected cells demonstrated sensitivity to EndoH (Figure 5B, lanes 13–14) indicative of ER retention. Consistent with the results observed in US3/US11 cells, the class I molecules were sensitive to EndoH in cells co-infected with US3+ and US11+-expressing viruses (Figure 5B, lanes 9–10). These virus-infected cells further demonstrate that US3 function dominates over US11 during the early phase of an HCMV infection upon expression of both viral proteins. Interestingly, increased amounts of ER-resident class I were recovered from Δ US2-11 infected cells (Figure 5B, lanes 7–8). The Δ US2-11 virus maintains expression of the US10 immune evasion gene product (Figure 4) that is known to bind and retain class I molecules within the ER (Furman et al., 2002), which may contribute to this effect. Sequential immunoprecipitation of US3 and US11 confirmed expression of these viral proteins at the 12 hour post-infection time point (Figure 5C). Collectively, our data from both stable cell lines and virus infection demonstrates that US3 does not enhance US11 function when these proteins are co-expressed, as was seen in US2/US3 cells (Noriega and Tortorella, 2009). Rather, it appears that US3 function dominates over US11-encoded function, allowing for limited class I degradation and enhanced retention of class I molecules within the ER.

4. DISCUSSION

A critical response to a viral pathogen involves the ability of the immune system to process viral peptides for presentation to specialized lymphocytes in order to hone the efforts of the host defense. MHC class I molecules, and thus class I-restricted antigens, are at the core of such an immune response. Successful human pathogens, such as HCMV, have developed strategies by which they circumvent immune recognition by inhibiting antigen presentation by MHC class I molecules (Tortorella et al., 2000b). Several independent loci within the HCMV genome contribute to down-regulation of surface class I molecules and protection from CTL recognition. The well-characterized HCMV immune evasins, US2 and US11, catalyze the extraction of the class I heavy chain component from the ER into the cytosol for proteasome-mediated degradation (Tortorella et al., 2000b). The HCMV US3 gene product retains class I molecules within the ER during immediate early time points of infection (Ahn et al., 1996). The temporal expression of US3, US2, and US11 suggests that these gene products function together to down-regulate class I molecules from infected cells. To that end, US2 and US3 indeed coordinate their activities towards the common goal of class I attenuation (Noriega and Tortorella, 2009). In this study, we demonstrate that HCMV US3 has the ability to differentially regulate US11-induced class I degradation. Stable expression of US3 and US11 led to an inhibition of dislocation and degradation of class I heavy chains (Figure 3). In fact, constitutive expression of US3 resulted in increased class I retention within the ER (Figure 2) and therefore decreased surface expression of class I molecules

(Figure 1). These findings were paralleled when fibroblasts were infected with AD169 recombinant viruses expressing US3 and US11 (Figure 5). During virus infection, class I could be down-regulated from the cell surface only upon expression of both US3 and US11 (Figure 5A). Further biochemical analysis revealed that this down-regulation during US3+/US11+ infection occurred due to enhanced retention of class I molecules within the ER (Figure 5B). The data suggests that HCMV US3 may tightly regulate the activities of both US2 and US11 during early time points of infection.

This fascinating interplay amongst US2, US3, and US11 is in agreement with published data for the immune evasion genes of the murine cytomegalovirus (MCMV). The three MCMV genes that affect class I antigen presentation (m04, m06, and m152) demonstrate analogous functions with their HCMV-encoded counterparts (US2, US3, and US11 proteins). m06 binds class I molecules and redirects them towards lysosomes for degradation (Reusch et al., 1999). m152 retains peptide-loaded class I within the *cis*-Golgi compartment (Ziegler et al., 2000). m04 has been shown to bind stably to class I and travel to the cell surface, where it may interact with lymphocytes in a protective manner (Kleijnen et al., 1997). The function of these immune evasion genes was initially described after their selective expression in culture. Interestingly, when their activities were studied in a systemic manner, through a complete set of mutant viruses that express different combinations of these genes, it was found that these viral proteins could act either synergistically or antagonistically (Wagner et al., 2002). These detailed studies demonstrated that, like HCMV US2 and US3, cooperativity could be observed between m152 and m06, with their combined impact on surface class I being greater than the impact of either alone. Interestingly, m04 could antagonize the down-regulation of class I imparted by m152, as we have reported for US3 and US11. Further studies have shown that m06 can increase the inhibition of CTL lysis achieved by either m152 or m04, while m04 functionally antagonized m152 for a subset of class I-restricted epitopes (Pinto et al., 2006). Our data further demonstrates that, for both HCMV and MCMV, the functional interplay between viral immune evasion genes is more complex during infection than initially believed.

Our data supports a model such that HCMV US3 may act as a molecular switch for virus-mediated class I degradation during early times of infection. This down-regulation could potentially alter the class I allelic profile of HCMV-infected cells. Analysis of class I levels in US3/US11 cells and virus infection demonstrates a down-regulation of total class I molecules (Figures 1 and 5). During the initial times of HCMV infection, the interplay of US3 with US11 may function to limit total class I molecules that can activate virus-specific CTLs. We have observed an efficient down-regulation of HLA-A2 alleles in US11-expressing cells (data not shown). However, US11 does not down-regulate HLA-B, HLA-C, HLA-E, and HLA-G alleles as efficiently as HLA-A alleles (Barel et al., 2006). Hence, the expression of US3 and US11 during infection could be capable of preventing a wider array of class I molecules from reaching the cell surface. Given that US3 is only expressed during the initial hours following infection, it is possible that US3, through assistance with US11, retains class I molecules in order to preferentially degrade properly folded class I molecules in an US2-dependent manner. This strategy suggests that all three gene products participate in class I down-regulation in a manner distinct from when they are expressed alone. Collectively, the data supports a paradigm that interplay between viral proteins during infection is likely a complex and regulated process that favors proliferation and minimizes host recognition. The interaction of viral proteins with each other and cellular factors creates an environment to efficiently manipulate cellular processes for the virus to limit immune detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CTL	cytotoxic T lymphocytes
ER	endoplasmic reticulum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HCMV	human cytomegalovirus
MHC	major histocompatibility complex
moi	multiplicity of infection
US	unique short

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HIGHLIGHTS

- HCMV US3 attenuates degradation of MHC class I molecules induced by US11
- Enhanced ER retention of class I molecules is evident during stable co-expression of US3 and US11
- Co-infection with US3- and US11-expressing viruses results in robust down-regulation of cell surface class I molecules
- While US3 enhances US2-mediated class I degradation, this viral protein antagonizes the function of the US11 gene product

Figure 1. Surface MHC class I molecules were down regulated in HCMV US3/US11 cells

Total cell lysates from U373, US3, US11, and US3/US11 cells were subjected to immunoblot analysis using anti-US3 (lanes 1–4), anti-US11 (lanes 5–8), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (lanes 9–12) antibodies. US3, US11, GAPDH, and molecular weight standards are indicated. **B.** U373, US3, US11, and US3/US11 cells were analyzed by flow cytometry using W6/32 monoclonal antibody followed by an anti-mouse immunoglobulin conjugated to Alexa 647. An immunoglobulin (IgG) control was included (shaded histogram). Plots are presented as normalized cell number versus surface class I fluorescence.

Figure 2. Class I molecules remain localized within the ER in US3/US11 cells

A. W6/32 precipitates (lanes 1–4) and cell lysates (lanes 5–8) from U373, US3, US11, and US3/US11 cells were subjected to an anti-class I heavy chain immunoblot. Class I heavy chains and molecular weight standards are indicated. The asterisk (*) represents a non-specific polypeptide. **B.** Cell lysates from U373, US3, US11, and US3/US11 cells were left untreated or treated with endoglycosidase H (EndoH) and subjected to an anti-class I immunoblot. Glycosylated (HC(+)-CHO) and deglycosylated (HC(-)-CHO) class I heavy chains, GAPDH, and molecular weight standards are indicated. **C.** U373, US3, US11, and US3/US11 cells plated onto coverslips were treated with W6/32 monoclonal antibody followed by incubation with a goat-anti-mouse immunoglobulin^{Texas Red}. A Hoechst stain was included to demonstrate nuclear staining.

Figure 3. US3 expression limits US11-mediated class I degradation

A. U373, US3, US11, and US3/US11 cells were metabolically labeled with ^{35}S -methionine for 15 minutes and chased up to 30 minutes. Samples were lysed in 0.5% NP40 and subjected to immunoprecipitation using HC10 (lanes 1–12), W6/32 (lanes 13–24), US11 (lanes 25–36) and US3 (lanes 37–48) antibodies. Class I heavy chains, US3, US11, and molecular weight standards are indicated. **B.** Total cell lysates from U373, US3, US11, and US3/US11 cells untreated or treated with ZL₃VS (2.5 μM , 16 hours) were subjected to an anti-class I (lanes 1–8) and GAPDH (lanes 9–16) immunoblots. Glycosylated (HC(+)-CHO) and deglycosylated (HC(-)-CHO) class I heavy chains, GAPDH, and molecular weight standards are indicated.

Figure 4. Schematic representation of HCMV mutants

The HCMV genome with the location of the unique long (U_L) and unique short (U_S) gene regions is shown on top. The region of interest is blown up in the maps below (not drawn to scale). The locations of the ORFs encoding US2, US3, US6 and US11 are shown by dark grey boxes. The Kan^R and Amp^R genes, used for BAC selection, and the FRT sites, used for constructing the BACs, are indicated.

Figure 5. US3/US11 induced down-regulation of MHC class I during HCMV infection

A. Cytofluorometric analysis of HLA-ABC surface expression was performed on human fibroblasts infected with the recombinant viral strains indicated. Cells were infected (moi: 5) for wild type, US3+, and US11+ viruses; co-infections were performed using an m.o.i. of 5 for each virus. At the indicated time points post infection, total MHC class I surface expression was measured using the HLA-ABC specific monoclonal antibody W6/32. The dashed vertical lines indicate HLA-ABC expression on cells infected with the Δ US2-11 mutant (set as 100%). **B.** Human lung fibroblasts infected with the recombinant viral strains were harvested at 12 hours post infection, metabolically labeled with 35 S-methionine for 15 minutes and subjected to immunoprecipitation using W6/32, followed by treatment with EndoH. Cells were infected at m.o.i. of 5 for wild type, Δ US2-11, US3+, and US11+ viruses; co-infections were performed using an m.o.i. of 5 for each virus. U373-US3/US11 cells were included as a control (Virus (-)). Glycosylated (HC(+))CHO and deglycosylated (HC(-))CHO class I heavy chains and molecular weight standards are indicated. **C.** Samples from **B** were sequentially immunoprecipitated for US11 (lanes 1–6), US3 (lanes 7–12), and PDI (13–18). US3, US11, PDI, and molecular weight standards are indicated.