Downregulation of MHC class I molecules by human cytomegalovirus-encoded US2 and US11

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CHAPTER 2

Amino acid composition of α1/α2 domains and cytoplasmic tail of MHC class I molecules determine their susceptibility to HCMV US11-mediated down-regulation

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Amino acid composition of α1/α2 domains and cytoplasmic tail of MHC class I molecules determine their susceptibility to human cytomegalovirus US11-mediated downregulation.

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During co-evolution with its host, human cytomegalovirus has acquired multiple defense mechanisms to escape from immune recognition. In this study, we focused on US11 that binds to MHC class I heavy chains and mediates their dislocation to the cytosol and subsequent degradation by proteasomes. To examine which domains of class I heavy chains are involved in this process, we constructed chimeric HLA molecules of US11- sensitive and - insensitive class I molecules (HLA-A2 and HLA-G, respectively). Pulse chase experiments were performed to evaluate protein stability and interactions between class I heavy chains and US11. Flow cytometry was employed to assess the effect of US11 on surface expression of the different chimeras. Our results indicate that the α1 and α2 domains of HLA molecules are important for the affinity of US11 association. However, the degradation efficiency seems to rely mostly on cytosolic tail residues. We found that the nonclassical HLA-G molecule is insensitive to US11-mediated degradation solely because it lacks essential tail residues. A deletion of the last two tail residues in full length class I already caused a severe reduction in degradation efficiency. Altogether, our data provide new insights into the mechanism by which US11 downregulates MHC class I molecules.

Human cytomegalovirus (HCMV) is a β-herpesvirus that causes serious disease and high mortality rates in immunocompromised persons, such as AIDS patients and organ transplant recipients. Besides this, HCMV is a notorious risk factor for congenital disorders if the mother undergoes primary infection during pregnancy. In immunocompetent hosts, HCMV infection generally gives benign but lifelong infections.

The host immune system is eluded by HCMV through a variety of defense mechanisms that it acquired while co-evolving with its host. Antigen presenting MHC class I molecules, which play a central role in detection and elimination of infected cells by CD8+ T cells and NK cells, represent a major target for viral evasion strategies. Cell surface expression of MHC class I molecules is affected by the concerted action of a set of proteins encoded within the unique short (US) region of the HCMV genome that are expressed along the different stages of viral infection. US3 is the first to be synthesized and prevents trafficking of newly synthesized MHC class I molecules by entrapping them in the ER [1]. Next, US2 and US11 come into play and induce proteolytic degradation of MHC class I molecules. Immediately after their synthesis and translocation into the ER, class I heavy chains are transported back into the cytosol where they are deprived of their N-linked glycan and subsequently degraded by proteasomes [2, 3]. At early and late times post infection, US6 prevents peptide loading of MHC class I molecules by blocking the Transporter associated with Antigen Processing (TAP) [4, 5]. Recently, another US-encoded gene, US10, has been reported to delay maturation of MHC class I molecules [6].

By downregulating surface expression of MHC class I molecules, HCMV infected cells may become a target for NK cells [7]. Several mechanisms have been described by which HCMV can sidetrack NK cells, including blocking expression of ligands that activate NK cells or allowing expression of ligands for inhibitory NK cell receptors. The unique long (UL) region encoded UL16 protein, expressed at an early stage of infection, can interfere with MICB/ ULBP1,2 - specific triggering of NK cell receptors [8]. At the same time, UL40 can promote surface expression of HLA-E by providing it with TAP-independent peptides, thereby supplying a ligand for certain inhibitory NK cell receptors [9]. At a late stage of infection, HCMV encodes a viral MHC class I homologue, UL18, which might serve as decoy for MHC class I at the cell surface [10]. The options to preserve inhibitory signals to NK cells could be further exploited by allowing migration to the cell surface for only a selected subset of MHC class I molecules. By preventing surface expression of the very potent antigen presenting HLA-A and -B locus products and allowing expression of

mostly nonclassical locus products that exhibit limited polymorphism, HCMV could escape killing by both CD8+ T cells and NK cells. Studies on US2 and US11 indicated that HCMV uses such discriminating mechanisms. From experiments with murine class I molecules, it is known that US2 and US11 each affect different subsets of MHC class I molecules [11]. More recent studies, including pulse chase and *in vitro* co-translation experiments, suggested that these viral gene products mainly affect HLA-A and -B allelic products, while HLA-C, -G and –E are able to escape degradation [12, 13, 14].

In the current study, we investigated how US11 distinguishes between different MHC class I locus products. At present, the domains of the class I heavy chains that are important for binding to US11, or that determine their sensitivity to US11-mediated dislocation and degradation have not been characterized. So far, it is clear that the cytoplasmic region of class I molecules is essential for US11-mediated degradation, although it does not seem to be essential for association with the viral protein [15].

Our approach to unravel the mechanism by which US11 distinguishes between different MHC class I alleles involved the use of chimeric HLA-A2/G molecules. HLA-A2, a classical MHC class I molecule, is known to bind to US11 and to be subjected to US11-mediated degradation [15]. For the nonclassical HLA-G, association with the viral protein has never been observed. Moreover, US11 has no effect on its half-life [12]. HLA-G molecules, which exhibit low polymorphism, are mainly found on trophoblast cells in placental tissues and could be involved in protecting these cells from maternal NK cell killing during pregnancy [16]. Although HLA-G molecules share many important features with other MHC class I molecules, it is important to note that the cytosolic tail consists of only 6 residues instead of 29-33 residues due to a premature stop codon in exon 6 [17]. By expressing the chimeric HLA-A2/G molecules in murine cells, we could specifically follow the fate of individual human class I molecules. We then evaluated the influence of US11 on their half-life and surface expression by pulse chase and flow cytometry.

MATERIALS AND METHODS

Cell lines

J26 cells (H-2^k murine Ltk$^-$ cells expressing human 2m) [23] and the Phoenix amphotropic retroviral producer cell line (generous gift H. Spits) were cultured in Dulbecco's Modified Eagle Medium (Life Technologies Inc.), supplemented with 10% FCS (Greiner), penicillin and streptomycin.

Antibodies

The following anti-MHC class I monoclonal antibodies (mAbs) were used for flow cytometry: 87G (directed against α1-domain of HLA-G, kind gift of Dr. D Geragthy), W6/32 (anti-human MHC I complex) [24], BB7.2 or MA2.1 (directed against α1-domain of HLA-A2) [25], B9.12.1 (anti-HLA-A, B, C, G) [26], R-Phyco-erythrin-conjugated goat anti-mouse IgG ((gαm–PE), Jackson, France) was used as second antibody. For immunoprecipitations HCA2 (against α1-domain of HLA-A and –E, -G), W6/32, US11(N2) (against the N-terminus of US11), and H68.4 (anti-transferrin receptor, (ZYMED)) Abs were used [24]. HCA2 was also used in western blot in combination with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (DAKO).

Construction of plasmids

An overview of the amino acid sequence of HLA-A2 and –G and of the different wild type and chimeric MHC class I constructs is depicted in Fig. 1. All MHC class I cDNA fragments were derived from either pcDNA-G1 (encoding HLA-G*01011) or pLUMC9901 (encoding HLA-A*0201) and were subcloned into the pcDNA3 vector (Invitrogen) [27, 24]. Chimeras CM#1, CM#2, CM#3 and CM#4 were constructed in a PCR applying the megaprimer method [28]. Amplifications were performed using *Pwo* polymerase (Eurogentec, Belgium. The HLA-A2short construct encodes an HLA-A2 heavy chain with a tail (-RRKSSD) as short as that of HLA-G (-RKKSSD). Mutations in the cytoplasmic tail of CM#1 were introduced with the QuickChange™ Site Directed Mutagenesis Kit (Stratagene). CM#1 mutants are depicted in Figure 3D. All constructs were fully sequenced to verify the absence of unwanted mutations.

Transfection

J26 cells have been transfected with the different chimeric MHC class I constructs using Effectene™ Transfection Reagent (Qiagen). 48 h later, stable transfectants were selected by using 0.3 mg/ml G418 (Geneticin; GibcoBRL). After a few days, cells were sorted by flow cytometry for expression of the introduced cDNA with BB7.2, B9.12.1 or 87G mAb.

Production of retrovirus and transduction.

A retroviral vector expressing both US11 and EGFP was constructed by ligating a US11-encoding

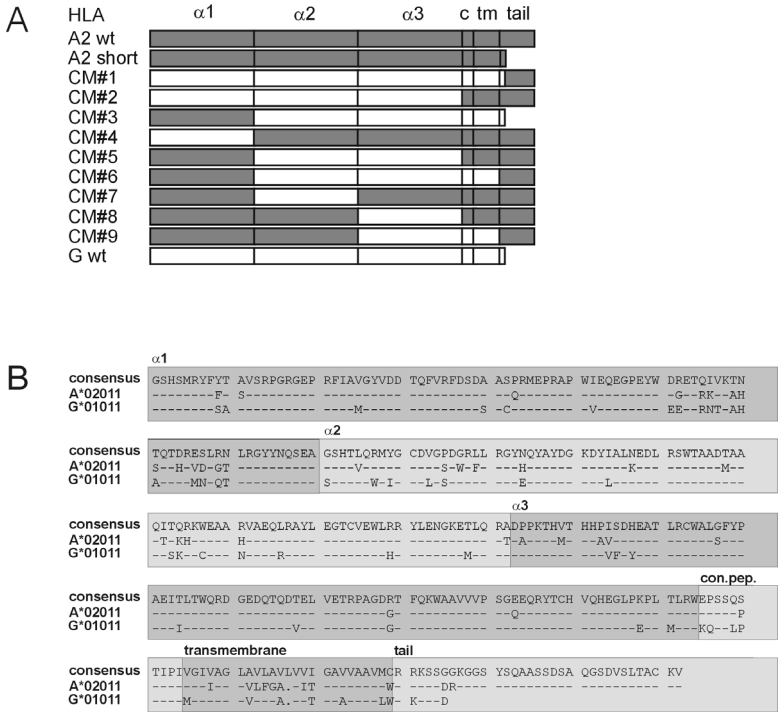

FIGURE 1. A) Schematic representation of the different domains of HLA-A2 and HLA-G that have been exchanged to generate the Chimeric Molecules (CM), c=connecting peptide, tm=transmembrane domain. **B)** Alignment of general HLA consensus sequence with HLA-A*02011 and HLA-G*01011 (www.ebi.ac.uk/imgt/hla/allele.html).

fragment into the pLZRS-*IRES*-EGFP vector [29]. This construct was used, together with the wild type EGFP expressing retroviral vector, to produce retrovirus by transfection of the amphotropic Phoenix packaging cell line with the calcium phosphate method. Transfected cells were grown under puromycin selection (2 μg/ml). 24 h before harvesting the retroviral supernatant, cells were grown in puromycin-free medium.

Cells were transduced with retrovirus using retronectin (Takara, Japan) coated dishes.

Vaccinia virus infections

J26 cells were infected with wild type or US11-expressing recombinant vaccinia viruses (rVV) (generous gift of Dr. J.Yewdell) at a multiplicity of infection of 10 PFU/cell for 45-60 min in a small volume of serum free culture medium at 37°C [30]. Metabolic labeling of infected cells was performed approximately $4^1/_2$ h after infection.

Flow cytometry

J26 cells were sorted for expression of human MHC class I with the appropriate primary antibodies and gαm-PE, using a Coulter EPICS-Elite (Coultronics) flow cytometer. After cell sorting 95-98% of the cells were positive for human MHC class I expression. A FACScan (Becton-Dickinson) flow cytometer was used to analyze surface expression of wild type/ chimeric HLA molecules as well as EGFP expression in cells transduced with retrovirus. Cells were stained as decribed in Kikkert et al [24]. Data were analyzed with the Cell Quest software package (Becton-Dickinson).

Prior to use of retroviral transduced cells in pulse chase experiments, cells were sorted for EGFP expression using a FACS Vantage flow cytometer.

Metabolic labeling, immunoprecipitation and SDS-PAGE

Metabolic labeling, immunoprecipitations and SDS-PAGE were performed as described by Kikkert et al. [24]. In brief, cells were starved in Met^-Cys^- medium at

37 °C, labeled with ^{35}S promix (Amersham), and chased in medium with excess amounts of L-cystine and L-methionine. Where indicated media were supplemented with 20mM proteasome inhibitor ZL_3H. Cells were lysed in NP40 lysis buffer containing leupeptin, AEBSF and ZL_3H. Immunoprecipitations were performed $\geq$ 2 h on precleared samples, with Abs coupled to protein A/G sepharose beads. After washing with NET buffer, samples were taken up in SDS sample buffer, boiled for 5 min and run on SDS-PAGE (10-15% gel). Gels were dried, exposed to a phosphor imaging screen, and analyzed by the Biorad Personal Molecular Imager FX .

Western blot

Samples were separated by SDS-PAGE (12.5% gel) and transferred onto PolyScreen PVDF membrane (NEN) using the BioRad Trans-blot semi-dry system. The membrane was blocked with 0.6 % BSA in TBST buffer (10 mM Tris pH 8, 150 mM NaCl, 0.05 % Tween-20). After incubation with HCA2 and gαm-HRP, bands were visualized using Western Lightning chemiluminescent substrate (Perkin Elmer) and Fuji Super RX film.

RESULTS

HLA-G becomes sensitive to US11-mediated degradation when its cytoplasmic tail is extended.

To identify the regions of MHC class I heavy chains that are responsible for US11-mediated down-regulation, we constructed chimeric HLA molecules that contain domains of HLA-A*02011 (HLA-A2) and HLA-G*01011 (HLA-G), Fig. 1A. An overview of the amino acid sequences of HLA-A2 and –G and the domains that have been exchanged to generate the different chimeric constructs is depicted in Figure 1B.

HLA-A2 is known to bind to US11 and to be subjected to US11-mediated degradation [15], whereas for HLA-G molecules neither association nor degradation has been observed [12]. To avoid cross-reaction of antibodies with endogenous class I molecules, experiments were performed in murine J26 cells. This cell line expresses human β2m to allow proper complex formation with the introduced HLA class I heavy chains [18]. After transfection with the different chimeras, J26 cells were transduced with US11/ EGFP- or control EGFP-expressing retrovirus.

To assess whether US11 similarly affects stability of HLA-A2 and HLA-G molecules in murine cells as in human cells, the transduced cells were metabolically labeled (Fig 2). In agreement with previous reports, HLA-G molecules remained stable over time in the presence of US11, while HLA-A2 molecules were degraded [12, 15]. The HLA-A2 signal disappeared over time in the absence of proteasome inhibitor, while in the presence of the inhibitor a deglycosylated degradation intermediate could be observed.

Next, the effect of US11 on surface expression of chimeric HLA-A2/G molecules was analyzed. HLA-G has a short cytoplasmic tail, compared to other MHC class I molecules. To analyze the effect of the tail size on US11-mediated modulation of surface expression, we extended the HLA-G construct with cytoplasmic tail residues of HLA-A2 (chimeric molecule (CM) #1). In addition, we constructed an HLA-A2 molecule with a cytoplasmic tail as short as that of HLA-G (HLA-A2short).

The different human MHC class I expressing cells were transduced with US11/EGFP- or control EGFP-expressing retrovirus. Since EGFP alone had no effect on MHC class I cell surface expression (data not shown), the EGFP negative population was used as reference for class I expression. The influence of

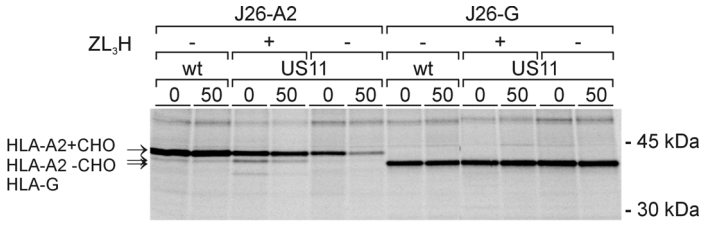

FIGURE 2. HLA-A2 is efficiently degraded in US11-expressing murine J26 cells, while HLA-G remains stable. J26 cells transfected with HLA-A2 (J26-A2) or HLA-G (J26-G) were transduced with control EGFP- or US11/EGFP expressing retrovirus. Cells were labeled (18 min) and chased (0 or 50 min), in the absence (-) or presence (+) of proteasome inhibitor (ZL_3H). HLA molecules were precipitated with HCA2 mAb. (-CHO= deglycosylated breakdown intermediate)

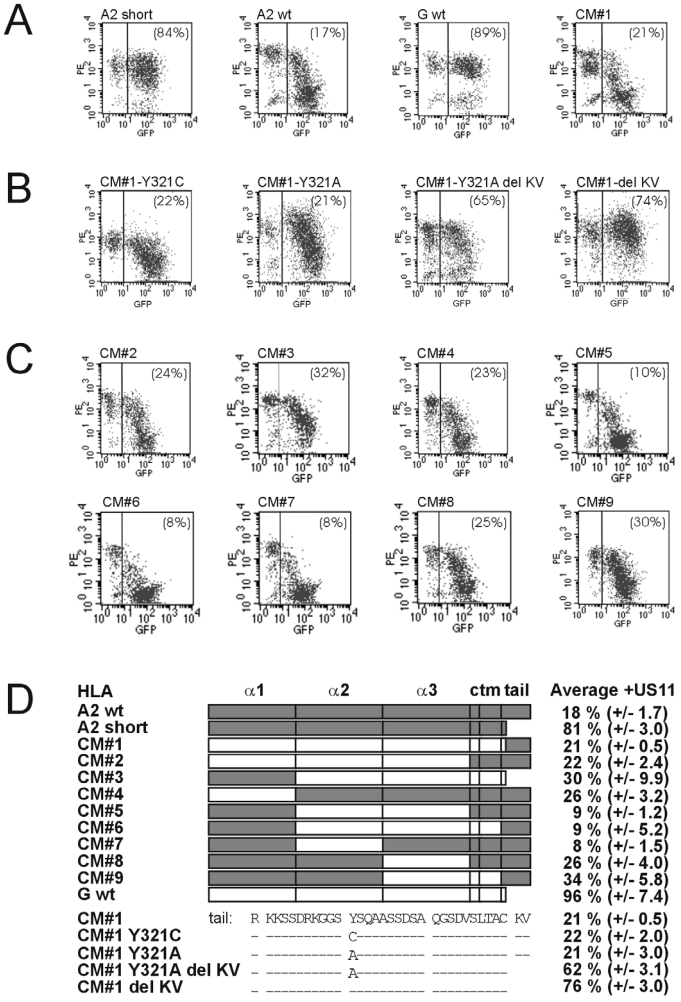

FIGURE 3. Effect of US11 on cell surface expression of wt type and chimeric MHC class I molecules. J26 cells were transfected with different HLA constructs and subsequently transduced with US11/EGFP expressing retrovirus. MHC class I molecules were labeled using W6/32, 87G (chimeras with α1 domain of HLA-G) or MA2.1 (chimeras with α1 domain of HLA-A2) mAbs followed by gαm-PE (Y-axis). US11 expression was measured indirectly by analyzing EGFP expression (X-axis). The effect of US11 expression on surface staining of HLA molecules is calculated as: mean PE fluorescence of US11 positive (EGFP+) cells divided by mean PE fluorescence of US11 negative (EGFP-) cells, times 100%. Contribution of: **A)** cytoplasmic tail **B)** sequence variation in the cytoplasmic tail **C)** ER-luminal domains and transmembrane region to susceptibility to US11-mediated downregulation of surface expression. **D)** Overview of chimeras and CM#1 tail mutants showing average percentage (~3 exp.) of their surface expression in US11+ cells.

US11 on cell surface expression of the chimeras was examined using flow cytometry, EGFP served as a marker for US11 expression (Fig. 3A).

The tailless constructs were hardly affected by the presence of US11: compared to the wild type situation, an average of 81% of HLA-A2short and 96% of HLA-G molecules were still able to reach the cell surface. In contrast, both full length constructs were downregulated by US11: only 18% of wild type HLA-A2 and 21% of CM#1 were expressed at the cell surface (Fig. 3A and D). Thus, the absence of most of the cytoplasmic tail residues is the main reason why this nonclassical HLA-G escapes downmodulation by US11.

Influence of cytoplasmic tail residues on down-regulation of MHC class I molecules.

In contrast to HLA-G, HLA-C alleles possess long cytoplasmic tails, but they are nevertheless considered insensitive to US11-mediated degradation [12]. For HIV an immune escape mechanism has been described which, like HCMV, appears to affect only a subset of HLA alleles. Nef internalizes HLA molecules with tyrosine at position 321 in the cytoplasmic tail (HLA-A, -B), but does not affect surface expression of class I molecules with C321 (HLA-C) [19]. HLA-A2 molecules with mutated Y321A are not affected by Nef [20].

To analyze the influence of amino acid composition of class I cytoplasmic tails on US11-mediated down-regulation, the following constructs were generated: CM#1 with a substitution of tyrosine 321 for alanine (CM#1-Y321A), or for cysteine (CM#1-Y321C); the Y321A mutation combined with a deletion of the last lysine and valine residues (CM#1-Y321A del KV) or the KV deletion alone (CM#1-del KV), (Fig. 3D).

A substitution of tyrosine 321 did not alter the sensitivity of class I to US11-mediated down-regulation, as CM#1, CM#1-Y321A and CM#1–Y321C were all similarly affected (Fig. 3B and D). However, CM#1-Y321A del KV was considerably less sensitive to US11, with an average reduction in cell surface expression to 62% for CM#1-Y321A del KV as apposed to 21% for CM#1. This effect can be ascribed to the KV deletion as CM#1-del KV shows a similar insensitivity to US11. Clearly, the extreme end of the cytoplasmic tail plays an important role in the downmodulation mediated by US11.

α1 and α2 domains of HLA molecules contribute to the sensitivity to US11-mediated down-regulation.

Besides the cytoplasmic tail region, the α1, α2, α3 or transmembrane regions of HLA molecules were exchanged (Fig. 3C and D). Since surface expression of HLA-A2 and CM#1 was affected by US11 similarly, major differences might not be expected for these chimeras. No clear role could be ascribed to the connecting peptide or transmembrane region of MHC class I in this experimental set up (compare CM#1 & 2, CM#5 & 6 and CM#8 & 9), nor to the α3 domain (compare CM#6 & 7). However, exchanging α1 and α2 ER-luminal domains did alter the sensitivity to US11: less than 10% of CM#5, CM#6 or CM#7 reached the cell surface in the presence of US11 whereas around 21-34% of the other full length HLA molecules was able to escape US11-mediated downregulation. Chimeras CM#5-7 carry the HLA-A2 α1 domain and at least the HLA-G α2 domain. Strikingly, the tailless construct CM#3, containing the same α1(A2)/ α2(G)-combination, was down-regulated by the action of US11, while HLA-A2short and HLA-G were not or only slightly affected (an average of 30% for CM#3, compared to 81% and 96% surface expression for HLA-A2short and HLA-G, respectively). By comparing flow cytometry data of CM#6 and CM#7 (both similarly affected with only 9% and 8% surface expression, respectively), we showed that it is the α2 domain of HLA-G that contributes to this increase in sensitivity. This is further supported by comparing data of CM#5 (10%) with CM#8 (26%) and CM#6 (9%) with CM#9 (34%) which only differ in their α2 domains. Altogether, these data indicate that both α1 and α2 domains of MHC class I molecules are involved in US11-mediated downregulation.

Decrease in cell surface expression of chimeric HLA-G molecules with an extended cytoplasmic tail is due to US11-induced degradation.

Since flow cytometry data only reflect the effect of US11 on cell surface expression of MHC class I molecules, we also evaluated its effect on their stability using pulse chase experiments.

Recombinant vaccinia virus (rVV) was used to introduce US11 into the cells. In parallel, a similar amount of cells were infected with wild type vaccinia virus. This way, a single cell line with a defined expression level of (chimeric) MHC class I molecules can be used to evaluate the effect of US11 expression. US11-rVV infects the cells with high efficiency and expresses high amounts of US11 protein after a few hours of infection [21]. Pulse chase experiments performed with rVV infected cells are depicted in Figure 4.

Class I heavy chains and murine transferrin receptor (mTfR) were immunoprecipitated from cell lysates, separated by SDS-PAGE and visualized using phosphor-imaging (Fig. 4A). Precipitated proteins were quantified and the amounts of MHC class I heavy chain (relative to the mTfR) are presented as a percentage of the wild type situation at time point zero (Fig. 4B). US11 expression is shown in figure 4C.

Figure 4 shows that the reduction of cell surface expression measured by flow cytometry correlates with degradation of MHC class I molecules in US11 positive cells. US11-mediated degradation was observed for HLA-G when its cytoplasmic tail was extended with HLA-A2 tail residues (CM#1), whereas a deletion of extreme tail end KV residues caused a severe decrease in degradation efficiency (compare CM#1 & CM#1-Y321A del KV). Exchange of the HLA-

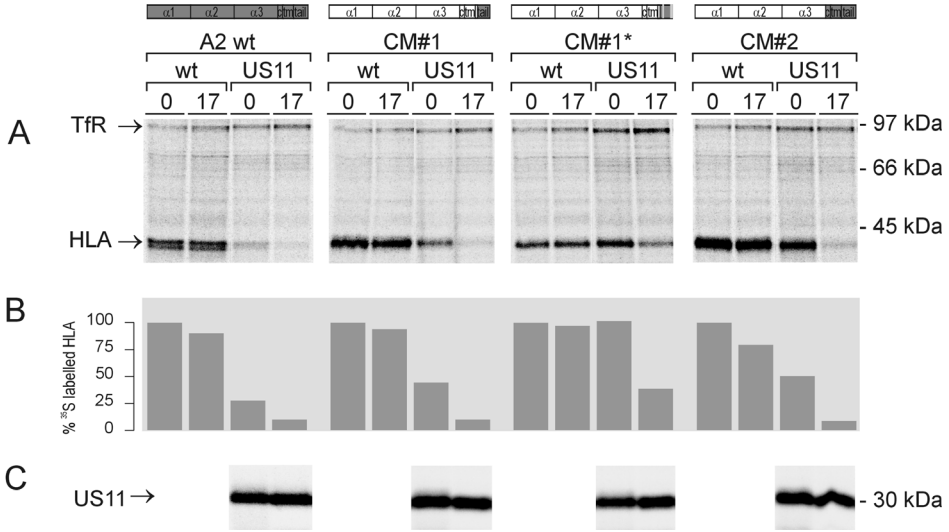

FIGURE 4. HLA-G chimeras with an extended cytoplasmic tail are susceptible to US11-mediated degradation. J26 cells transfected with HLA-A2, CM#1, CM#1 Y321A del KV (CM#1*) or CM#2 were infected with wt or US11-expressing vaccinia virus. Cells were labeled (8 min.), 4½ hours after infection, and chased for 0 or 17 min. **A)** Transferrin receptor (TfR) and HLA molecules were immunoprecipitated from cell lysates with anti-TfR (H68.4) and anti-heavy chain (HCA2) mAbs, respectively, separated by SDS-PAGE and visualized using a phosphor-image screen. **B)** Amount of precipitated HLA protein (relative to the TfR and t=0 wt VV infected sample), given as percentage. **C)** After immunoprecipitation of HLA/TfR molecules, US11 was immunoprecipitated using anti-US11(N2) Ab.

A2 connecting peptide/transmembrane region did not alter class I heavy chain stability (compare CM#1 & CM#2).

In addition, these data provide information on elimination rates of HLA molecules. Whereas similar amounts of HLA-A2, CM#1 and CM#2 heavy chain were eliminated within 17 min of chase, there was a difference in the time course over which these molecules were cleared: immediately after the pulse (chase t=0), almost three quarters of HLA-A2 heavy chains was eliminated, versus half of the CM#1 & CM#2 material. These findings suggest that chimeras containing ER-luminal domains derived from HLA-G are somewhat less susceptible to US11-mediated degradation than HLA-A2 molecules. Ultimately, similar amounts of HLA-A2 and HLA-G molecules with tails as long as that of wild type HLA-A2 are degraded and thereby withheld from the cell surface.

US11 causes intracellular retention of a tailless HLA-G chimeric molecule with the α1 domain of HLA-A2.

Tailless HLA-A2short and HLA-G molecules are relatively insensitive to US11-mediated down-regulation (Fig. 3D). Surprisingly, the tailless HLA-G chimera, in which the α1 domain of HLA-G has been replaced with that of HLA-A2 (CM#3), proved to be much more sensitive to US11 (Fig. 3D). How can this be explained? Is the α1 (A2) / α2 (G) combination advantageous for a strong association with US11 (or other component(s) involved in the process of US11-mediated dislocation) and causing intracellular retention, or is this chimera (CM#3) being dislocated and degraded even though it lacks a long cytoplasmic tail?

To address these questions, pulse chase analysis was performed with J26-CM#3 cells (Fig. 5). The stability of CM#3 heavy chains was hardly affected by US11 (Fig. 5A & B). In US11-expressing cells, some decrease in the amount of CM#3 heavy chains could be observed at 90 min of chase, but this seems to be US11-independent since a similar decrease is observed in control cells. Furthermore, CM#3 seemed incapable of forming normal (W6/32-reactive) complexes with β2m when US11 was present (Fig. 5C). In agreement with flow cytometry data, HLA-G and HLA-A2short heavy chains were capable of forming stable complexes with β2m in the presence of US11 (data not shown).

Fig. 5D shows that anti-US11 antibody, besides US11, also immunoprecipitated a protein with a mobility in SDS-PAGE similar to that of CM#3. The protein was identified as CM#3 by blotting the same anti-US11 immunoprecipitate onto PVDF-membrane,

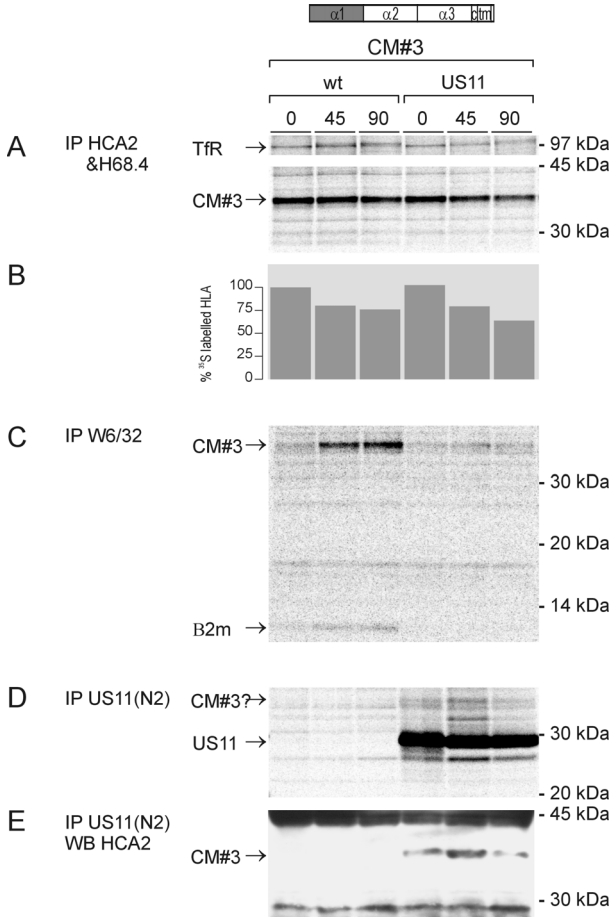

FIGURE 5. The tailless chimera of HLA-G with the α1 domain of HLA-A2 (CM#3) associates with US11 and is retained intracellularly. J26 cells transfected with HLA-CM#3 were infected with wt or US11- expressing vaccinia virus. Cells were labeled (15 min), 4 ½ hours after infection and chased for 0, 45 or 90 min. Cell lysates were split in three**. A)** Immunoprecipitation of class I heavy chains (HCA2) and transferrin receptor (H68.4) **B)** quantified amounts of HLA (relative to TfR and t=0 wt VV infected sample). Immunoprecipitation **C)** of MHC I complexes (W6/32) or **D**) of US11 (anti-US11(N2)). **E)** Half of the anti-US11(N2) sample was run on a separate gel for a Western blot and stained with HCA2 and gαm-HRP Abs.

followed by staining with anti-human heavy chain antibody (Fig. 5E).

Altogether, these data show that downregulation of CM#3 at the cell surface is not caused by protein degradation, but instead results from intracellular retention, presumably due to its interaction with US11.

DISCUSSION

In this study, we explored which domains of MHC class I heavy chains are responsible for association with the HCMV-encoded US11 protein, and subsequent dislocation and degradation. Using a set of chimeric HLA molecules of US11-sensitive and US11-insensitive class I molecules (HLA-A2 and HLA-G, respectively), we found that a long cytoplasmic tail was required for efficient US11-induced degradation of both classical HLA-A2 as well as nonclassical HLA-G heavy chains. In addition, we demonstrated that the lysine and valine residues at the extreme end of the cytoplasmic tail, together with the amino acid composition of both α1 and α2 ER-luminal domains play an important role in US11-mediated MHC class I downregulation.

US11 exclusively degrades HLA-A2 molecules with long cytoplasmic tails [19]. Using an HLA-G/A2 chimera with a long cytoplasmic tail (CM#1), we demonstrated that HLA-G is insensitive to US11-induced degradation solely because it lacks essential cytoplasmic tail residues. We then tested whether alterations in the amino acid composition of the tail influenced the efficiency of downmodulation. We found that a substitution of tyrosine residue 321, a critical residue for selective HIV Nef-mediated internalization of MHC class I molecules, had no influence. Interestingly, a deletion of only two residues (lysine and valine) at the extreme end of the cytoplasmic tail made class I molecules a lot less susceptible to US11-mediated downregulation. We are currently investigating the exact role of these residues with respect to selectivity of the US11-mediated dislocation and degradation process.

Our data also indicate that the amino-acid composition of both α1 and α2 luminal domains influences US11-mediated MHC class I down-regulation. Pulse-chase experiments with wild type HLA-A2 and chimeric HLA-G/A2 molecules with long cytoplasmic tails showed that ER-luminal domains determine the efficiency of the degradation process: wild type HLA-A2 molecules were degraded faster than the chimeric HLA molecules in which ER-luminal domains consisted of HLA-G residues (CM#1, CM#2). Note that although US11 needed more time to induce degradation of these chimeras, eventually similar amounts of wild type HLA-A2, CM#1 and CM#2 were eliminated and therefore withheld from the cell surface.

Our flow cytometry experiments showed that the α1 and α2 ER-luminal domains predominantly determine downregulation, rather than the α3 domain. The combination of the HLA-A2 α1 domain with at least the α2 domain of HLA-G in different long tailed chimeras resulted in an even more efficient downregulation than that of wild type HLA-A2 cell surface expression. Unexpectedly, this same combination even led to a major downregulation of a tailless chimera (CM#3), whereas surface expression of HLA-G and HLA-A2short were not or only slightly affected. Pulse chase experiments indicated that US11 is incapable of mediating degradation of this tailless chimera, presumably because it lacks essential cytoplasmic tail residues. The observed reduction in surface expression is more likely due to intracellular retention by US11. In agreement with this hypothesis, complexes of US11 and class I heavy chains could be detected. In addition, formation of W6/32 reactive molecules was strongly reduced in the presence of US11. US11 may therefore interfere with the folding of class I heavy chains and/or their association with β2m, although it cannot be excluded that US11 binding blocks the W6/32 epitope.

Based on our data, we propose the following model: US11 could first associate with newly synthesized MHC class I heavy chains via their α1/α2 domains. The affinity or association/dissociation rates might depend on the amino acid composition of these luminal domains while the amino-acid composition of the cytoplasmic tail would be critical for efficient dislocation to the cytosol and subsequent degradation by the proteasome. According to this model, the unexpected downregulation of the tailless chimeric HLA-G molecule in which the α1 domain has been replaced with that of HLA-A2 (CM#3) could be explained by a high affinity interaction, but slow dissociation rate between US11 and CM#3. The interaction between US11 and HLA-G could be so brief that it does not affect cell surface expression. The association with HLA-A2short could be somewhat longer as US11 does have a slight effect on surface expression of this tailless molecule (reduction to 81% cell surface expression). The interaction with HLA-G and HLA-A2 is nevertheless long enough to induce their dislocation and degradation if they possess the essential tail residues. The amino acid composition of the cytoplasmic tail then determines the efficiency with which this dislocation/degradation process takes place.

Our data seem to be in contrast with a previous report [12]. Based on *in vitro* experiments in which different MHC class I heavy chains (HLA-A2, -C, -G) were co-translated with either US2 or US11, Schust et al. suggested that these viral proteins could only associate with HLA-A2 and that the stability of HLA-C and HLA-G was due to a lack of association. It is possible that an interaction of HLA-G with US11 could not be detected in these experiments as the interaction might be very transient and difficult to detect under the conditions used. However, we do believe that this interaction takes place as tailless class I molecules can associate with US11 and the CM#1 molecule (HLA-G with an extended cytoplasmic tail) can be degraded.

Like US11, US2 is capable of inducing degradation of MHC class I molecules. Our data indicate that these two viral proteins interact with distinct ER-luminal domains of class I heavy chains. We showed that

binding to US11 involves the $\alpha1/\alpha2$ domains of class I molecules. Experiments performed with US2 and this same set of chimeras did not reveal involvement of the combination $\alpha1/\alpha2$, but pointed towards a combination of $\alpha2/\alpha3$, as both wt HLA-A2 and CM#5 were downregulated around 5 fold more efficiently than CM#7 (unpublished results). These data are in agreement with the previously published crystal structure of a soluble HLA-A2/US2 complex, which demonstrated association of US2 with ER-luminal $\alpha2/\alpha3$ domains [22]. Association of US2 and US11 with distinct domains of class I molecules may contribute to a broader defense of HCMV, as US2 and US11 together could attack a wider variety of MHC I molecules.

Altogether, our data provide new insights in the mechanism by which US11 downregulates MHC class I molecules. It will help unraveling the criteria for selective modulation of different MHC class I subsets involved in NK and T cell recognition.

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