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

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

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voor mijn ouders

Contents

Abbreviations		**8**
Chapter 1	Introduction	**9**
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. *Eur J Immunol. 33: p1707-1716 (2003)*	**27**
Chapter 3	HCMV-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation. *J Immunol. 171: p6757-6765 (2003)*	**41**
Chapter 4	Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2-and US11-mediated degradation. *Submitted*	**57**
Chapter 5	Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. *Biochem J. 358: p369-377 (2001)*	**71**
Chapter 6	Human cytomegalovirus-encoded US2 and US11 target unassembled MHC class I heavy chains for degradation. *Mol Immunol. : in press (2005)*	**85**
Chapter 7	Summary and Discussion	**99**
Nederlandse samenvatting		**107**
Curriculum vitae		**111**
Publications		**113**

Abbreviations

aa	amino acid
β2m	β2-microglobulin
BiP	Immunoglobulin binding protein
Cnx	calnexin
Crt	calreticulin
CTL	cytotoxic T lymphocyte
DTT	dithiothreitol
EGFP	enhanced green fluorescent protein
EndoH	endoglycanase H
ER	endoplasmic reticulum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
gαm	goat anti-mouse
HC	heavy chain
HCMV	human cytomegalovirus
HLA	human leucocyte antigen
IFN	interferon
IL	interleukin
IRES	internal ribosomal entry site
kDa	kilodalton
mAb	monoclonal antibody
MHC	major histocompatibility complex
MOI	multiplicity of infection.
NK cell	natural killer cell
NP40	nonidet P-40
PDI	protein disulfide isomerase
PE	phycoerythrin
prot K	proteinase K
TAP	transporter associated with antigen processing
TM	transmembrane
TNF	tumor necrosis factor
TX-100	triton X-100
UL	unique long
US	unique short
wt	wild type
ZL_3H	carboxybenzyl-leucyl-leucyl-leucinal
ZLVS	carboxyl benzyl-leucyl-leucyl-leucyl vinylsulfone

CHAPTER 1

Introduction

Introduction

Defense mechanisms against viruses

In healthy individuals, the immune system is able to limit the damage in many viral infections. When a viral particle manages to invade the body by crossing physical barriers such as the skin and mucus membranes, it becomes subject to detection and elimination by several immune effector cells. As viruses cannot replicate on their own, they must infect cells in order to exploit the host cell's machinery for the production of progeny and to enable further virus spread.

Host cell entry can be blocked by antibody-producing B cells, which specifically recognize foreign structures on the viral particle. The antibodies cannot only sterically hinder viral entry, but they can also induce elimination of the viral particles via different pathways. These include complement activation, antibody-dependent cell-mediated cytotoxicity and phagocytosis [1,2]. Two subtypes of B cells arise after activation of B cells, antibody producing plasma cells and memory B cells. The latter subtype is capable of mounting an immediate high-affinity response upon renewed contact with the antigen.

Once virus particles are inside host cells, T cells come into play. They can recognize foreign antigen fragments displayed in the context of major histocompatibility complex (MHC) molecules at the cell surface [3,4]. There are two classes of MHC molecules, which differ in structure, expression pattern and source of antigenic peptides.

MHC class I molecules are found on all nucleated cells and mainly present peptides derived from endogenously synthesized proteins. Circulating $CD8^+$ cytotoxic T cells (CTLs) inspect antigenic fragments displayed by MHC class I molecules. In case of infection, CTLs recognizing foreign antigens can, upon activation, kill such cells.

Expression of MHC class II molecules is restricted to antigen-presenting cells, i.e. B cells, macrophages and dendritic cells. Class II molecules display peptide fragments, derived from an exogenous source of proteins (e.g. viral capsid proteins), for inspection by $CD4^+$T cells. Upon recognition of foreign peptides, these T cells provide help to $CD8^+$ T cells to elicit a cytotoxic response. In addition, $CD4^+$ T helper cells are required for the maintenance and functionality of memory $CD8^+$ T cells [5].

The presentation of exogenous peptides is not completely restricted to MHC class II molecules, as peptides derived from internalized proteins can also enter the MHC class I pathway [6,7]. The ability to cross-present seems to be limited to a specific subset of antigen presenting cells, mainly $CD8^+$ dendritic cells [8]. In addition, endogenous proteins can gain acces to the MHC class II pathway via autophagy [9].

Besides an interaction between MHC molecules and their specific receptors, a ligation between accessory molecules on the antigen presenting cell and counter-receptors on the T cell (co-stimulation) is required for T cell activation.

Natural killer (NK) cells are also capable of sensing virally infected cells, but can only do so by virtue of altered expression levels of certain native surface proteins, including MHC class I molecules. The activation of NK cells, and the subsequent release of cytotoxins, is controlled by a set of NK cell receptors. Depending on the type of receptor, inhibitory or activating signals are ignited upon binding ligands on the potential target cell. If inhibitory receptor signaling fails to quench the effect of engagement of activating receptors, the NK cell will be triggered to kill its target cell.

This thesis will further focus on MHC class I molecules and their implication as ligands for T and NK cell receptors in the context of human cytomegalovirus infection.

Human cytomegalovirus

Human cytomegalovirus (HCMV) is found in human populations worldwide and can infect 60–90% of individuals, depending on the population studied. HCMV is a member of the *Herpesviridae*, which are known to establish a life-long relationship with their hosts. HCMV infection is usually asymptomatic, but can cause disease in an opportunistic manner. In immunocompromised individuals, such as AIDS patients and organ transplant recipients, uncontrolled HCMV reactivation can cause severe illness or death [10,11]. Additionally, HCMV is a notorious risk factor for congenital birth defects. In most cases, such clinical manifestations are found in mothers who undergo primary rather than recurrent infections [12].

In immunocompromised individuals, HCMV can induce pathology in nearly every organ system.

In vivo, HCMV can infect endothelial cells, epithelial cells, monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, fibroblasts, and hepatocytes [13,14]. *In vitro,* all tested vertebrate cell types were susceptible to HCMV infection [15]. Common HCMV-associated illnesses include retinitis (which may cause blindness), hepatitis, encephalitis, pneumonia, and bowel disease [16].

Following primary infection, HCMV persists in a latent state in cells of the myeloid lineage, with intermittent viral reactivation and shedding from mucosal surfaces, and containment by the host immune response [17]. Another potential cell type latently or persistently infected by HCMV are vascular endothelial cells [18]. Interestingly, HCMV infection has different effects on the functioning and survival of these various types of host cells. In macrovascular aortic endothelial cells HCMV infection is not lytic and results in the accumulation of significant amounts of extracellular, but not intracellular virus. In addition, the cell cycle is not inhibited by HCMV and cells continuously release infectious virus. This is in contrast with the rapid, lytic infection of brain microvascular endothelial cells and human foreskin cells [18]. It also differs from HCMV infection in monocyte-derived macrophages, which results in non-lytic accumulation of intracellular virus and lack of extracellular virus [19].

At present, the viral and host mechanisms of HCMV latency and reactivation are unclear, but the virus may be reactivated by cellular differentiation, and hormonal or cytokine (TNFα) stimulation.

HCMV particles can be found in all body fluids and can be transmitted by multiple means, including aerosol droplets, sexual contact, nursing, transplantation and blood transfusion [20].

HCMV has a large double stranded DNA genome ($\geq$230 kb), encoding approximately 192 proteins [21]. The size of the genome varies among HCMV strains. Compared to low-passage clinical HCMV isolates, the laboratory-adapted AD169 and Towne strains lack 22 and 19 viral genes, respectively [22].

In infected cells, viral replication occurs according to a cascade of three consecutive phases, called immediate early (IE), early (E), and late (L). The IE gene products play an important role in regulating the expression of viral E and L genes, as well as of cellular genes. E and L viral gene products include viral functions associated with viral DNA replication and virus packaging. The viral genome also encodes several proteins involved in immune evasion strategies, dealing with various immune effector mechanisms of the host. These include interference with MHC class I and II antigen presentation to $CD8^+$ and $CD4^+$ T cells, NK cell activity, cytokine and chemokine signalling, and apoptosis [23-37].

It is evident that these mechanisms are not exploited in a way that is life-threatening to the immunocompetent host. Several studies support important roles for B, T and NK cells in protection against HCMV disease [38-44]. However, these immune evasion strategies may prevent complete eradication of the virus and contribute to the ability of HCMV to cause lifelong persistent infections.

Before discussing the immune escape mechanisms exploited by HCMV to interfere with MHC class I surface expression, an introduction on MHC class I complex formation and the different functions of the various MHC class I locus products will be given.

MHC class I molecules

MHC class I molecules consist of a 43 kDa heavy chain, a 12 kDa light chain (β2-microglobulin, β2m) and a peptide of 8-10 amino acids in length (see Figure 1). The heavy chains are type 1 transmembrane proteins consisting of three ER-lumenal /surface exposed domains (α1-3), a connecting peptide, transmembrane (TM) region and a cytoplasmic tail. The α1-3 domains bind β2m non-covalently. The α3 domain and β2m show homology with immunoglobulin domains and have similar folded structures, whereas the α1 and α2 domains fold together into a single structure consisting of two segmented α-helices lying on a sheet of eight antiparallel β-strands. The folding of the α2 and α3 domains creates a long groove, which is the site where peptide antigens bind. Together with peptide, this highly polymorphic MHC class I region determines T-cell antigen recognition. A single binding site can bind a wide variety of peptides with high affinity, but there are some sequence restrictions. The peptides that can bind to a given MHC variant have the same or very similar amino acid residues at two or three particular positions, called anchor residues, along the peptide sequence. The amino acid side chains at these positions insert into pockets of the MHC class I molecule that are lined by polymorphic residues of the heavy chain. Additional amino acid positions within the peptide, called secondary anchors, can also influence MHC binding. These anchor residues differ

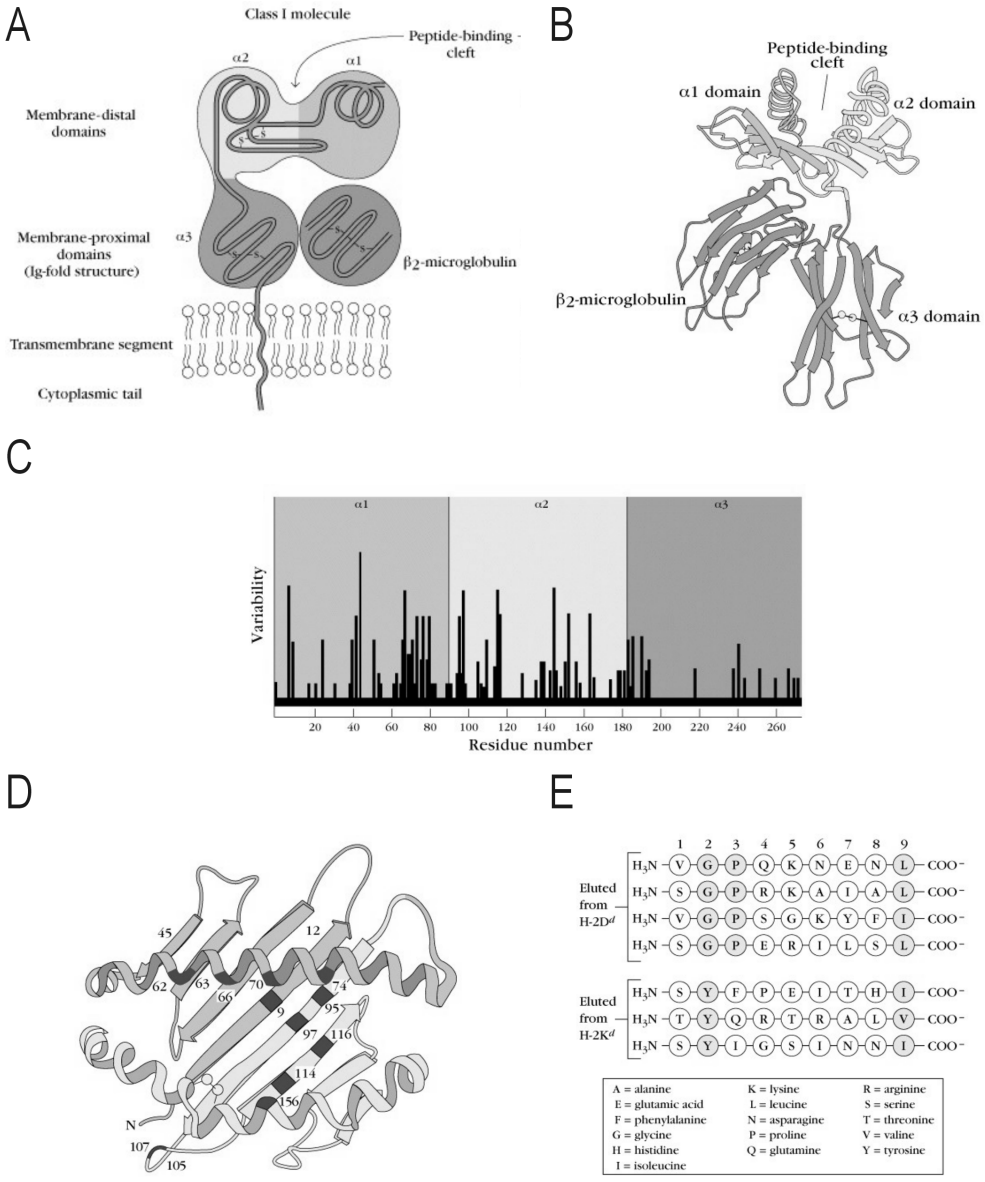

Figure 1. Features of MHC class I molecules. A) Schematic diagram of an MHC class I molecule showing the external domains, the transmembrane segment and the cytoplasmic region. The peptide binding cleft is formed by the membrane distal 1 and 2 domains of class I. **B)** Representation of the human class I HLA-A2 molecule determined by x-ray crystallographic analysis. The -strands are depicted as thick arrows and the-helices as helical ribbons. Disulfide bonds are represented as two interconnected spheres. **C)** A plot of the variability in the amino acid sequence of allelic class I molecules in humans demonstrates that the variable residues are clustered in the 1 and 2 domains. **D)** Representation of the 1 and 2 domains as viewed from the top of a class I molecule, showing the cleft consisting of a base of anti-parallel beta strands and sides of -helices. **E)** Examples of anchor residues in nonameric peptides eluted from two class I MHC molecules. Anchor residues (grey) tend to be hydrophobic amino acids and interact with the class I MHC molecule. *(Adopted from J. Kuby, Immunology 4th ed., W.H Freeman)*

for peptides binding different alleles of MHC class I molecules. Altogether, this ensures the display of a wide variety of peptide antigens for $CD8^+$ T cell inspection.

MHC class I assembly and interactions with components of MHC class I antigen presentation pathway

The folding and assembly into mature trimeric complexes involves a series of events and requires the action of several accessory molecules (see Figure 2). As HCMV-encoded proteins intervene at different stages of this process, the MHC class I antigen presentation pathway will be discussed in more detail.

MHC class I heavy chains encode a signal peptide, which directs insertion into the ER during translation. Once in the ER, the signal sequence is cleaved off by a signal peptidase, while oligosaccharyl transferase equips the HC with an N-linked oligosaccharide. These free HCs are soon found in association with the general ER chaperones BiP [45] and membrane bound calnexin, the latter of which has lectin-like activity [46,47]. BiP transiently binds to many newly synthesized proteins. Misfolded proteins and unassembled subunits are bound by BiP in a prolonged fashion. [48,49]. Binding of calnexin is regulated by glucose trimming of nascent N-linked oligosaccharides [50]. Calnexin generally binds proteins with mono-glucosylated (Glc1Man9-7GlnNAc2) oligosaccharides [51]. Calnexin and BiP predominantly associate with free MHC class I heavy chains and the assembly with β2m abolishes the interaction of the heavy chain with these chaperones [45,52,53]. Before binding the light chain, heavy chains also interact with ERp57, a member of the protein disulfide isomerase (PDI) family, who are involved in disulfide bond oxidation, reduction and isomerization reactions [54-56]. Mature MHC class I molecules harbor three intra-molecular disulfide bridges, the formation of which is likely to be mainly assisted by ERp57. After binding β2m, MHC class I molecules are found in association with another, soluble ER chaperone with lectin-like activity, calreticulin [57,58]. Like calnexin, calreticulin binds to proteins with Glc1Man9-7GlnNAc2 N-linked oligosaccharides [59,60]. MHC class I molecules than become associated with the peptide-loading complex, which besides calreticulin includes ERp57, tapasin, and the transporter associated with antigen processing (TAP). Tapasin mediates the interaction of class I with TAP [57,58,60]. The structure of MHC class I-peptide complexes suggests that oxidation of the cysteines in the HC α2-domain is a prerequisite for stable peptide binding, a process likely facilitated by ERp57 [61]. The peptides are generated from endogenous proteins by cytosolic proteasomes (large protease complexes, discussed in more detail in a later section) and may be further trimmed by aminopeptidases before and after translocation into the ER via TAP [62,63]. In the ER, peptides are loaded onto HC-β2m heterodimers. These trimeric HC-β2m-peptide complexes then dissociate from the loading complex and are released into the secretory pathway [64].

MHC class I variants

In humans, a single type of light chain (B2m) is found that is complexed with one of multiple types of heavy chains, HLA-A, B, C, E, G, of which up to two different allelic forms can be expressed. They are encoded by separate clusters within the MHC region of the human genome, located on chromosome 6. This region also encodes other proteins involved in antigen presentation (e.g. MHC class II, TAP1, TAP2, LMP genes). The light chain is encoded by a gene located on another site, on chromosome 15. The various types of heavy chains differ from each other in several ways, as will be discussed below.

Polymorphism

One outstanding difference among class I haplotypes, is the degree of polymorphism that can be found in the world population. To date, 372 different alleles have been reported for HLA-A, and 661 for HLA-B. HLA-C shows somewhat less variation with 190 different alleles. Polymorphism of HLA-E and HLA-G is very limited, with 5 (HLA-E) and 15 (HLA-G) different alleles described to date (IMGT/HLA database, [65].

Tissue distribution / surface expression

Tissue distribution and cell surface levels differ for the various locus products. Most host cells express HLA-A, B, C and E alleles, whereas HLA-G expression is restricted to the thymus and certain placental tissues, e.g. trophoblast cells. The trophoblast cells at the maternal-fetal interface lack surface expression of HLA-A and -B alleles [66,67]. Outside this immune-privileged site, the surface levels of HLA-A, -B, and -C alleles may vary between cell types [68,69]. Several aspects that influence MHC class I (surface) expression levels will be discussed below.

Regulation of MHC class I gene transcription

HLA class I genes have different constitutive and cytokine-induced expression patterns, which can be

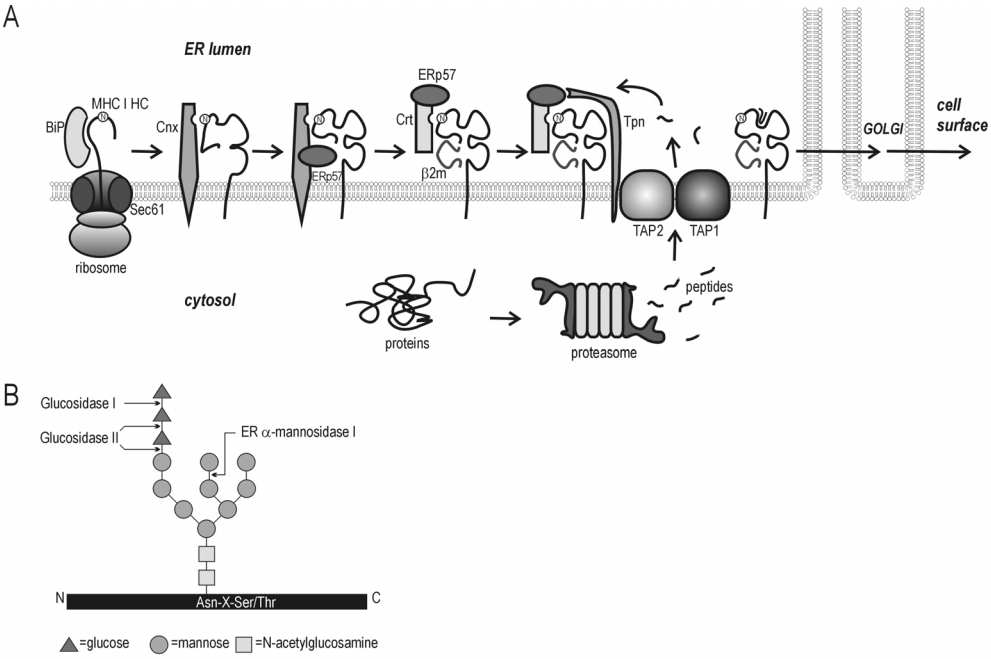

Figure 2. MHC class I assembly. A) Model for folding and complex formation of MHC class I molecules and interactions with components of the class I antigen presentation pathway (see text for further description). Calnexin (Cnx), calreticulin (Crt), tapasin (Tpn). The N-linked glycan is marked "N". **B)** N-linked glycans are added to proteins in the ER as "core oligosaccharides" that have the structure shown. These are bound to the polypeptide chain through an N-glycosidic bond with the side chain of an asparagine that is part of the Asn-X-Ser/Thr consensus sequence. The three glucoses are removed by glucosidase I and II, and terminal mannoses by one or more different ER mannosidases. Association of glycopeptides with Cnx/Crt is dependent upon the presence of a single terminal glucose residue bound to the $GlcNAc_2Man_9$ glycan precursor. *(Adapted from ref.142*

attributed to differences in their promoter regions. [69-71]. The cytokines TNF-α, IFN-γ and IFN-β can increase average MHC class I surface levels up to 10-fold [69]. The HLA-G promoter region differs from other MHC class I genes, as some of the conserved regulatory boxes have been deleted or altered, including the IFN regulatory sequence [70,72]. In addition, the cytokine IL10 has been reported to selectively upregulate HLA-G expression [73]. Cell type-specific factors may contribute to differential locus expression as well [68,69].

MHC class I complex formation and stability

The levels of the different MHC class I molecules that are found at the cell surface are determined by transcript levels, as well as by the success of stable complex formation, and turnover rates. Generally, surface levels of HLA-C locus products are rather low, about 10% of the average level of HLA-A and -B molecules [74-76]. Several explanations have been proposed, including lower transcript levels, lower assembly rates for HLA-C heavy chains with β2m, and an inefficient supply of high-affinity HLA-C-specific peptides. [71,77,78]. Variation between MHC class I molecules in binding components of the peptide loading machinery, tapasin/TAP, may also contribute to differences in MHC class I surface levels. Experiments with wild type and mutant HLA-A2 molecules indicated that residues 132-134 in the α2 domain are important determinants for association with TAP. HLA-A2 T134K/S132C mutants do not associate with tapasin/TAP and are released into the secretory pathway much faster (as indicated by reaching Endo H resistance) than their wild type counterparts [79]. There is no sequence variation in this particular region. Residues 114 and 116, which are polymorphic, were shown to be responsible for differences in peptide-loading complex interactions [80-84]. Class I molecules that associate inefficiently with intracellular chaperones may generally demonstrate

lower peptide ligand binding affinities and render the heterotrimeric MHC class I complexes formed less stable. Once at the cell surface, tyrosine or di-leucine motifs present in most MHC class I molecules can function as endocytosis signals. HLA-G misses potential endocytosis signals and its half-life has been shown to be almost twice that of HLA-A2 molecules [85].

Peptide binding / repertoire

HLA-A/B and HLA-C/G/E differ in their ligand-binding capabilities: HLA-A/B molecules are promiscuous, whereas the other class I molecules bind a limited number of ligands. HLA-C locus products, for instance, have a preference for peptides that are poorly transported into the ER, and that require a 10-fold higher peptide concentration for release into the secretory pathway [78,86-88]. HLA-G binds many self-peptides with a defined motif and its peptide binding diversity is estimated to be about five fold lower than that of HLA-A [89,90]. The most common HLA-E ligands are nonamers derived from signal sequences of other HLA class I molecules [91,92].

Special features / alternative splice variants

Due to a premature stop codon in exon 6, HLA-G has a relatively short cytoplasmic tail (6 residues) compared to other HLA class I molecules (29-33 residues) [93]. Consequently, HLA-G not only lacks potential endocytosis signals present in other class I molecules, but it also gains an ER retrieval/retential signal with the dilysine residues positioned at -4 and -5 from the C-terminus. This motif can mediate recycling of assembled HLA-G molecules between the ER and the *cis*-Golgi [94,95]. High affinity peptide binding seems to be required to end the recycling and allow egress to the cell surface [85].

Primary HLA-G mRNA transcripts are differentially spliced, giving rise to multiple isoforms [96]. Only the full length isoform (HLA-G1) is expressed at the cell surface [97]. In addition to this membrane-bound isoform, a soluble (secreted) isoform was detected in placental tissues (sHLA-G1 /HLA-G5). Soluble HLA-G1 is translated from a transcript with a retained intron 4, which introduces a premature stopcodon after the α1-3 domain encoding sequence. This isoform can associate with β2m [98,99].

Ligands for T and NK cell receptors

MHC class I molecules can be ligands for cytotoxic T cell receptors, as well as for NK cell receptors. Through recognition of MHC-peptide complexes, $CD8^+$ T cells can kill infected target cells. HLA-A and B molecules are the restriction elements in the majority of CTL responses, although there are also some examples of HLA-C, -G and -E molecules presenting antigenic peptides to CTLs [100-103]. This may very well explain the high degree of polymorphism that is particularly described for HLA-A and –B alleles.

Unlike for CTLs, the distinction between self and non-self antigens in the context of MHC class I molecules is not the major restriction element for triggering of NK cell lysis. This trigger is determined by the presence or absence of NK cell receptor ligands on the surface of the target cell. The total input of several types of ligands, including MHC I molecules, and their engagement with both inhibitory and activating NK receptors controls NK cell cytolysis [104,105]. Many MHC class I molecules can contribute to the regulation of NK cell functioning. Other NK cell receptor ligands include several molecules that are distantly related to MHC class I molecules, e.g. MIC A/B, UL16 binding protein (ULBP) 1/2/3, and CD155. An overview of currently known NK cell receptors and ligands is presented in Table 1 [106,107].

A particular NK cell clone can express up to nine different receptors, which together determine the activation status of the cell. These receptors can be displayed on overlapping subsets within the total NK cell population, and the repertoire of expressed receptors is heterogeneous in different individuals [108,109]. Some of these receptors are also found on other immune effector cell types. Variation in surface expression of only one type of MHC class I molecule can already make a difference for cell survival.

Table 1. Natural killer cell receptors and their ligands

Name of receptor	Type of signal	Ligand(s)
KIR2DL1 (p58.1)	inhibitory	HLA-C^{Lys80}
KIR2DL2 (p58.2)	inhibitory	HLA-C^{Asn80}
KIR2DL4 (p49)	inhibitory	HLA-G
KIR3DL1 (p70)	inhibitory	HLA-Bw4
KIR3DL2 (p140)	inhibitory	HLA-A3, A11
LIR1/ILT2	inhibitory	HLA-A,-B,-C,-E,-F,-G
LIR2/ILT4	inhibitory	HLA-A,-B,-C,-E,-F,-G
CD94/NKG2A	inhibitory	HLA-E
KIR2DS1 (p50.1)	activating	HLA-C^{Lys80}
KIR2DS2 (p50.2)	activating	HLA-C^{Asn80}
KIR2DS4 (p50.3)	activating	unknown
CD94/NKG2C	activating	HLA-E
P40/LAIR1	inhibitory	unknown
p75/AIRM1	inhibitory	unknown
NKp30 (1C7/NK-A1)	activating	unknown
NKp44	activating	unknown
NKp46	activating	unknown
NKp80	activating	unknown
NKG2D	activating	MICA/B, ULBP1-3
2B4	activating	CD48
DNAM-1 (CD226)	activating	PVR; nectin-2 (CD155)
KLRF1 (NKp80)	activating	unknown

Surface expression of HLA-E was shown to be sufficient to either inhibit NK cells expressing CD94/NKG2A or to enhance killing by cells expressing CD94/NKG2C [110]. Likewise, HLA-G expression has been shown to protect HLA class I deficient targets from NK cell mediated lysis, through engaging inhibitory NK cell receptors [111,112]. The selective expression of only HLA-C, -E, and –G alleles on cells at the fetal maternal interface may help to protect them from maternal cytotoxic T cell and NK cell attack.

T cell and NK cell escape mechanisms of HCMV

Several immune escape mechanisms exploited by human cytomegalovirus seem to focus on prevention of detection and elimination by cytotoxic T cells and NK cells.

HCMV and escape from cytotoxic T cell killing

During latent infections, HCMV viral gene expression is limited which helps to avoid immune surveillance by cytotoxic T cells. During active infection, HCMV exploits another mechanism to avoid display of viral antigens, that is by down-regulating MHC molecules. 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 [30,113]. 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 into the cytosol where they are deprived of their N-linked glycan and subsequently degraded by proteasomes [37,114]. 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) [29,33,115]. Another US-encoded gene, US10, has been reported to delay maturation of MHC class I molecules [25].

HCMV and NK cell escape

A complete reduction of MHC class I expression could have serious consequences for the survival of the virus, as cells lacking MHC class I surface molecules are more susceptible to NK-cell attack [24]. Several proteins encoded within the Unique Long (UL) region of the HCMV genome (UL16, UL18, UL40) appear to protect infected cells against NK-cell lysis. They either block expression of ligands that activate NK cells (UL16) or allow expression of ligands that can inhibit NK-cell triggering (UL18, UL40) [34].

UL16, expressed at an early stage of infection, interferes with MICB/ ULBP1, 2 -specific triggering of activating NK cell receptors (NKG2D) by down-regulating their ligands [116]. At the same time, UL40 can promote surface expression of HLA-E by providing it with TAP-independent peptides, thereby supplying a ligand for the inhibitory CD94/NKG2A receptor, which is found on most NK cells [102]. At a late stage of infection, HCMV encodes a viral MHC class I homologue, UL18, which serves as decoy for MHC class I at the cell surface and can bind the LIR1 inhibitory NK cell receptor [117]. Recently, a new early/late HCMV-encoded gene product has been identified, UL141, which prevents surface expression of CD155, a ligand for the activating NK cell receptor CD226 [36]. It is important to note that this UL141 gene is present in various clinical HCMV strains, whereas it is lost in laboratory AD169 and Towne strains [21,22,36].

Alternative ways to preserve inhibitory signals to NK cells could include a more selective down-regulation of MHC class I surface expression. If the specificity of the US proteins were such that those MHC class I molecules that present viral antigens (mostly HLA-A and -B alleles) are affected predominantly, HCMV could escape both T cell and NK cell killing.

Selectivity and degree of MHC class I down-regulation by HCMV

The success of immune escape by HCMV from both T cell and NK cell attack through modulation of MHC class I surface expression is likely to be influenced by the efficiency as well as by the specificity of the different viral evasion proteins.

The degree of down-modulation that can be accomplished likely depends on the balance of target MHC class I and US protein levels. As mentioned before, MHC class I surface levels can be upregulated by cytokines. The expression levels of the US proteins vary during the course of infection and may also depend on viral load [118]. A higher amount of viral particles can lead to a more severe reduction in MHC class I surface expression [119]. As mentioned in previous sections, the total cellular MHC class I pool is rather heterogeneous. Besides contributing to a more synergistic effect on down-regulation of one particular MHC class I locus product, expression of different US proteins may also account for a broader effect on the total pool of MHC class I products.

Several laboratories have studied allelic differences between MHC class I molecules with respect to sensitivity to US2, US3, US6, and US11 [29,33,67,102,113,115,119-126].

Our studies were focused on the specificity of MHC class I down-regulation by US2 and US11. In addition, we aimed to characterize the precise regions in MHC class I alleles that determine sensitivity or resistance to US2 and US11. The approach for US2 was based on previous results and on crystal structure data from a soluble HLA-A2/β2m/US2 complex (see Figure 3) published by Gewurz et al. [123].

Dislocation and degradation of MHC class I molecules

In the ER, US2 and US11 bind newly synthesized MHC class I heavy chains and target them to the cytosol for subsequent degradation by proteasomes [31,37]. Retro-transport is a mechanism commonly used for the disposal of improperly folded and unassembled ER lumenal proteins, including MHC class I heavy chains. The exact requirements for dislocation of these membrane-anchored proteins from the ER into the cytosol are still relatively unknown. However, there are indications that certain ER chaperones may be involved.

ER quality control and ER-associated degradation of incompletely folded or assembled MHC class I molecules

When properly folded heterotrimeric HC-β2m-peptide complexes are formed, the MHC class I complex is released of all auxiliary molecules as it follows the secretory pathway. But until this point, several quality control mechanism are active to prevent surface expression of malfolded or unassembled class I heavy chains. For instance in the absence of one of the components of the complex, e.g. in case of a defect in β2m expression, no MHC class I molecules can be detected at the cell surface [127-130]. If the supply of peptides is hampered by TAP inhibition, or loading is obstructed by an impaired interaction of TAP and HC's in the absence of tapasin, then the surface expression of class I molecules is also severely reduced [57,131].

In β2m and TAP-deficient cell lines, incompletely folded and assembled MHC class I molecules are removed from the ER and released into the cytosol, where they are degraded by proteasomes [132]. The exact mechanism by which molecules are dislocated remains elusive, but there are indications that ER

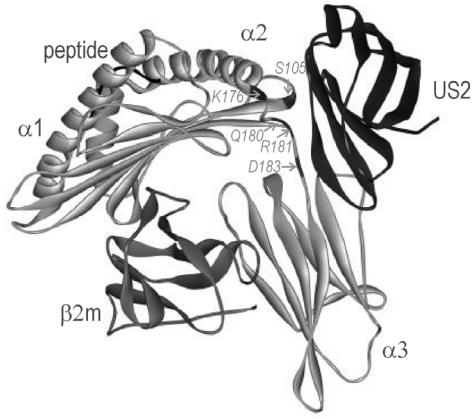

Figure 3. Crystal structure of a soluble US2/HLA-A2/β2m/Tax complex. Crystal structure of a soluble US2/HLA-A2/β2m/Tax complex, as determined by B. Gewurz et al. Data for this image were derived from the PUBMED Protein Data Base (reference 1IM3) and visualized as solid ribbon using WebViewerLite. HLA-A2 (light grey), β2m (darker grey), US2 (darkest grey) and Human T lymphotropic virus type I Tax peptide (LLFGYPVYV, black). Amino acid residues in HLA-A2 that make direct contact with US2 are depicted in dark grey and are marked with their corresponding position numbers in the class I heavy chain.

chaperones such as BiP and calnexin may play a role in this process. BiP retains many misfolded proteins in the ER, including unassembled HC's [45,133,134]. Studies involving Kar2p (the yeast homologue of BiP) and glycoprotein CPY*, have linked the ATPase activity of BiP with release of malfolded proteins into the cytosol [135]. The release from BiP and retro-transport of unassembled Ig L chain, a soluble nonglycosylated protein, has been found to be tightly coupled with proteasome activity [136]. It is unclear whether these effects of BiP on dislocation are restricted to malfolded soluble ER proteins. Besides this, BiP has been implicated to play a role in the unfolded protein response (UPR), as sensor of accumulating misfolded proteins. UPR directs the upregulation of a number of stress-related proteins (likely involved in disposal processes), including BiP [137,138]. Like BiP, Calnexin retains incompletely assembled MHC class I HC's in the ER by using its cytoplasmic tail [52,139]. Surface expression of MHC class I heavy chains in a β2m-deficient cell line could be restored by introducing calnexin with a truncated tail [52].

As mentioned before, trimming of the N-linked precursor oligosaccharide Glc3Man9GlcNAc2 in the ER yields Glc1Man9-7GlnNAc2, which enables association with calnexin and calreticulin [51]. A folding

and quality control process is then initiated that consists of cycles of deglucosylation by glucosidase II, release from the chaperone, reglucosylation by the folding sensor UDP-Glc:glycoprotein glucosyltransferase, and re-association with calnexin and calreticulin [140]. Glycoproteins that achieve proper folding are no longer recognized by glucosyltranferase and leave this cycle. Differential mannose trimming by ER-mannosidases I and II has been proposed to signal the degradation of terminally misfolded glycoproteins [141-143]. Inhibitors that prevent mannose trimming were found to inhibit degradation of many defective glycoproteins [141,143-147]. Loss of the mannose residue that is the acceptor for glucosyltranferase leads to release from the reglucosylation cycles involved in the rescue of improperly folded glycoproteins and association to the putative mannose lectin EDEM [148-150]. The glycoproteins are then dislocated to the cytosol, deglycosylated and degraded by the proteasome [151].

Ubiquitin and proteasomal degradation

The turnover of many cellular proteins is regulated by a process, which involves initial earmarking via the ubiquitin system and subsequent degradation by proteasomes (see Figure 4). This earmarking involves the linkage of ubiquitin, a highly conserved protein of ~8.5kDa, to the substrate protein through an isopeptide bond between the C-terminal glycine (Gly76) of ubiquitin and the ε-NH_2 group of a lysine residue in the target protein. Protein ubiquitination involves a cascade reaction with subsequent activities of three different types of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) [152,153]. Polyubiquitin chains are formed by the attachment of additional ubiquitin moieties via Gly76, to Lys48 of the next ubiquitin moiety. Only poly-ubiquinated proteins are recognized as substrates for proteasomal degradation. A fourth type of enzyme (E4) has been postulated to function as multi-protein chain assembly factor [154].

Ubiquitin-mediated proteolysis is an important mechanism for the regulation of many cellular processes, as it mediates the turnover of cell cycle regulators, tumor suppressors and growth modulators, transcriptional activators and inhibitors, cell surface receptors, in addition to misfolded proteins. This requires a substrate specific mode of action for the E1-3 enzymes. E1 enzymes are highly conserved and so far only a single ubiquitin-activating enzyme (Uba1) has been found in yeast and humans. The ubiquitin-conjugating (ubc) enzymes are part of a homologous family of proteins. To date, 13 different E2 enzymes have been described in yeast, and at least 18 in humans. Likely, E3 enzymes contribute most to the biological specificity of the ubiquitin system, with hundreds, if not thousands of often structurally unrelated enzymes. These include Skp/Cullin/F-box (SCF), SOCS-box, Anaphase Promoting Complex (APC), HECT family, RING finger domain, U-box, VBC and Parkin-like E3 ligases.

Proteasomes, found both in the nucleus and cytosol, are large multi-subunit complexes, made up of a 20S core particle and one or two regulatory "cap" complexes (19S or 11S/PA28)[155]. The 20S barrel-shaped core particle consists of four stacked rings: two identical outer α-rings, and two identical inner β-rings. Each of these rings contain seven different subunits. The catalytic activity is provided by 3 subunits (called X, Y and Z) in the β-rings. The 19S cap is composed of 17 subunits, 9 of which are part of a base connected to the α-ring, and 8 of which are found in a structure referred to as the lid. This cap recognizes poly-ubiquitinated proteins and is believed to mediate entry into the narrow pore of the 20S core particle by unfolding the substrate and widening the entrance via the α-ring. Immuno-modulatory cytokines such as IFN-γ can alter the composition of the proteasome. IFN-γ promotes expression of the regulatory PA28/11S complex, which reportedly enhances proteasome activity[156,157]. In addition, IFN-γ induces the incorporation of LMP2, LMP7 and MECL-1 subunits into the β-rings of the 20S core particle, instead of the standard X, Y, and Z subunits[158-160]. Proteasomes harboring IFN-γ subunits are therefore referred to as immunoproteasomes. Immunoproteasomes may enhance the specificity of the proteasome and promote the generation of peptides presented by MHC class I molecules.

Dislocation of ER-lumenal proteins and involvement of cytosolic factors

It is still largely unknown how dislocation of ER-lumenal substrates is accomplished. Proteasomes may play an active role in the extraction of ubiquitinated substrates. In mammalian cells ~10% of the proteasomes are located at the cytosolic face of the ER membrane, and proteasomal activity appear to be required for the extraction of several transmembrane proteins [161-163].

More recently, the cytosolic AAA ATPase p97-Ufd-Npl4 complex was linked to the dislocation of ER proteins. This complex seems to be involved in the

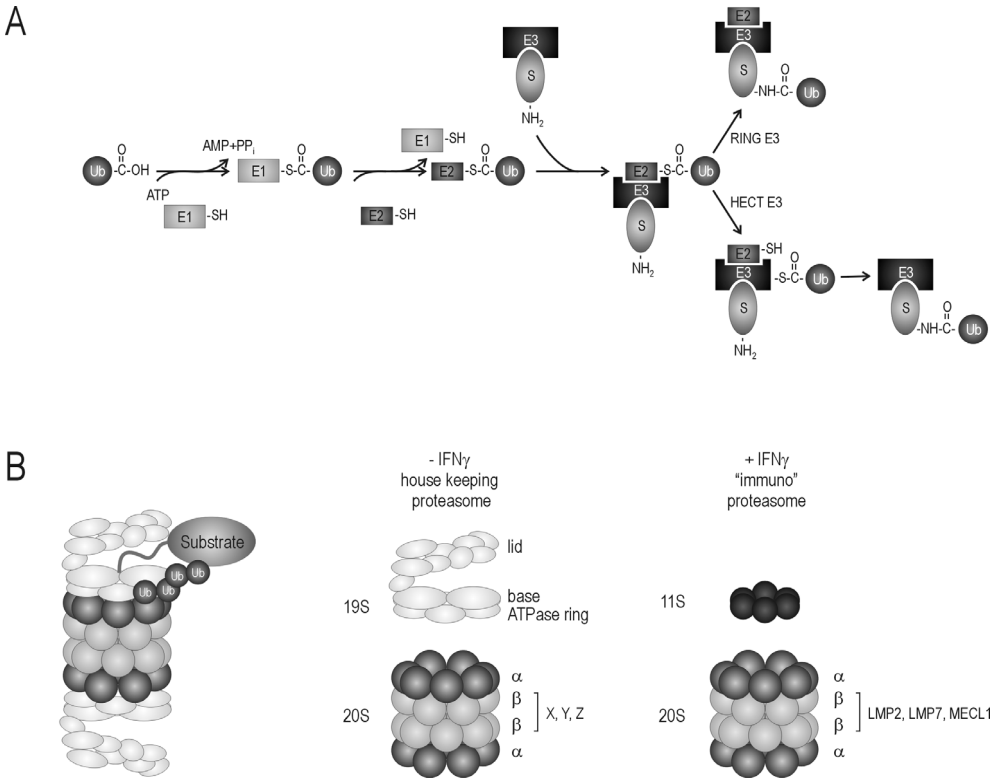

Figure 4. Ubiquitin-dependent protein degradation. A) Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s, which might or might not have substrate (S) already bound. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HECT domain followed by transfer to substrate (as shown) or to a substrate-bound multi-ubiquitin chain. For RING E3s, current evidence indicates that ubiquitin might be transferred directly from the E2 to the substrate. **B)** After linkage of a poly-ubiquitin chain onto the substrate protein, the earmarked protein can be recognized by the 19S (PA700) cap, which is composed of 12 non-ATPase-like (Rpn) and 6 ATPase-like regulatory particles (Rpt). The 19S cap is associated with the 20S proteasome complex, in which degradation is taking place. The 20S core has a barrel-shaped structure of four stacked rings, each comprising 7 subunits. The outer rings harbor subunits and the inner rings subunits. The proteolytic activity resides in a pair of three different subunits (called X, Y and Z). In the presence of IFN, the composition of proteasomes is altered. In this case IFN--inducible subunits LMP2, LMP7, and MECL1, replace X, Y and Z subunits. IFN- also induces expression of the proteasome regulator 11S (PA28), a hexameric complex consisting of 11S +. Different combinations of 20S cores with caps exist, as the 20S core particle can go with two 19S or two 11S caps, or with a mix of the two. *(Adapted from A.M. Weissman, Nature Reviews 2001*

final membrane detachment of partially dislocated substrates [164,165]. It is a hexameric ring complex, that senses both poly-ubiquitinated as well as non-ubiquitinated proteins [165]. It likely acts in concert with other (ER-membrane anchored) proteins exerting a more substrate-specific role.

In studies of US11-mediated degradation of MHC class I heavy chains, Derlin1 (the mammalian equivalent of yeast Der1), was identified as an additional component involved in dislocation [166-168].

Scope of this thesis

The studies described in this thesis focus on strategies exploited by human cytomegalovirus to escape immune detection by its host through modulation of surface expression of MHC class I molecules by US2 and US11 (reviewed in Chapter 1). US2 and US11 can both target newly synthesized MHC class I molecules for proteasomal degradation. This can prohibit the display of viral antigens on the surface of infected cells and frustrate inspection by cytotoxic T cells. However, the absence of surface class I molecules could also alert NK cells and trigger cytolysis by these immune effector cells.

If the specificity of the viral US2 and US11 proteins was such that those MHC class I molecules presenting viral antigens (mostly HLA-A and -B alleles) are preferentially affected, these US proteins could contribute to an escape of HCMV-infected cells from both T cell and NK cell killing. In this thesis, we investigated whether US2 and US11 indeed display selectivity in their down-regulation of MHC class I locus products (Chapters 2-4).

Although at first sight the mode of action of US2 and US11 seems very similar, it is unlikely that HCMV encodes two different proteins with identical function. We evaluated if US2 and US11 could act complementary to each other. They could do so in various ways, e.g. by targeting different MHC class I subsets for degradation, by using different pathways for degradation of class I molecules, or by acting at different stages in the folding and assembly process of MHC class I molecules. Therefore, we evaluated several of these aspects.

First of all, we explored the structural requirements of MHC class I molecules to become targets for US2 (Chapters 2 and 4) or US11 (Chapters 3 and 4) using various chimeric and mutant HLA class I molecules.

Ubiquitin serves as an earmark to target proteins for proteasomal degradation and it could also provide a handle for pulling ER protein into the cytosol. Therefore, we also assessed the role of the ubiquitin system in US11-mediated dislocation of MHC class I molecules (Chapter 5).

Finally, we evaluated whether US2 and US11 can act in early stages of MHC class I folding and assembly, by targeting unassembled HCs for degradation (Chapter 6).

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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

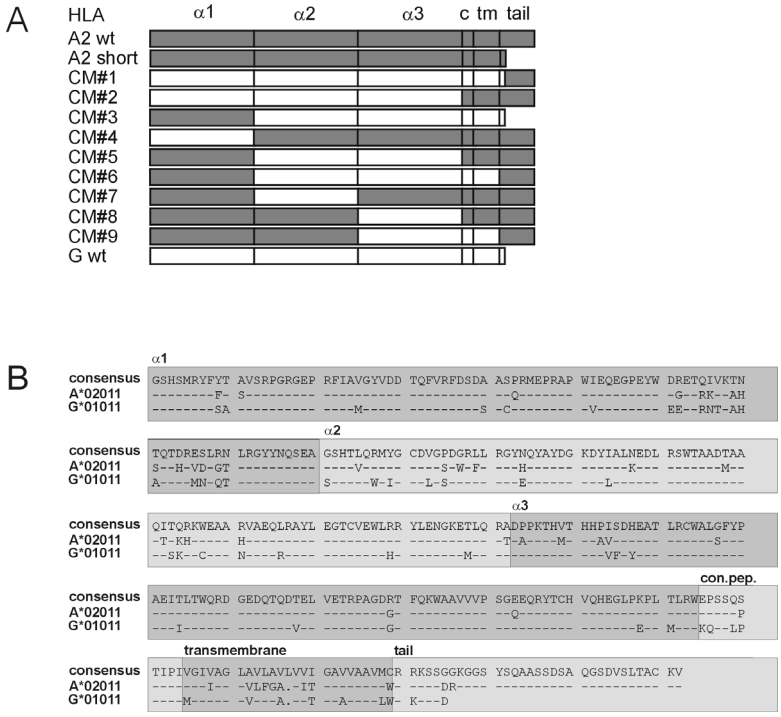

FIGURE 1. A) Schematic representation of the different domains of HLA-A2 and HLA-G that have been exchanged to generate the Chimeric Molecules (CM), c=connecting peptide, tm=transmembrane domain. **B)** Alignment of general HLA consensus sequence with HLA-A*02011 and HLA-G*01011 (www.ebi.ac.uk/imgt/hla/allele.html).

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

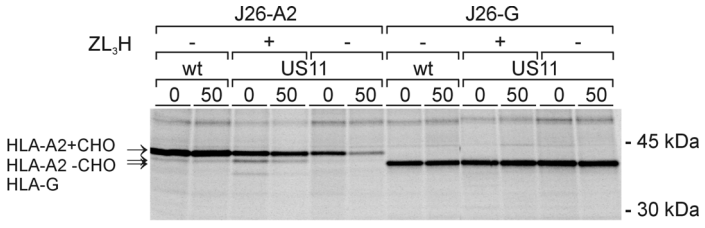

FIGURE 2. HLA-A2 is efficiently degraded in US11-expressing murine J26 cells, while HLA-G remains stable. J26 cells transfected with HLA-A2 (J26-A2) or HLA-G (J26-G) were transduced with control EGFP- or US11/EGFP expressing retrovirus. Cells were labeled (18 min) and chased (0 or 50 min), in the absence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

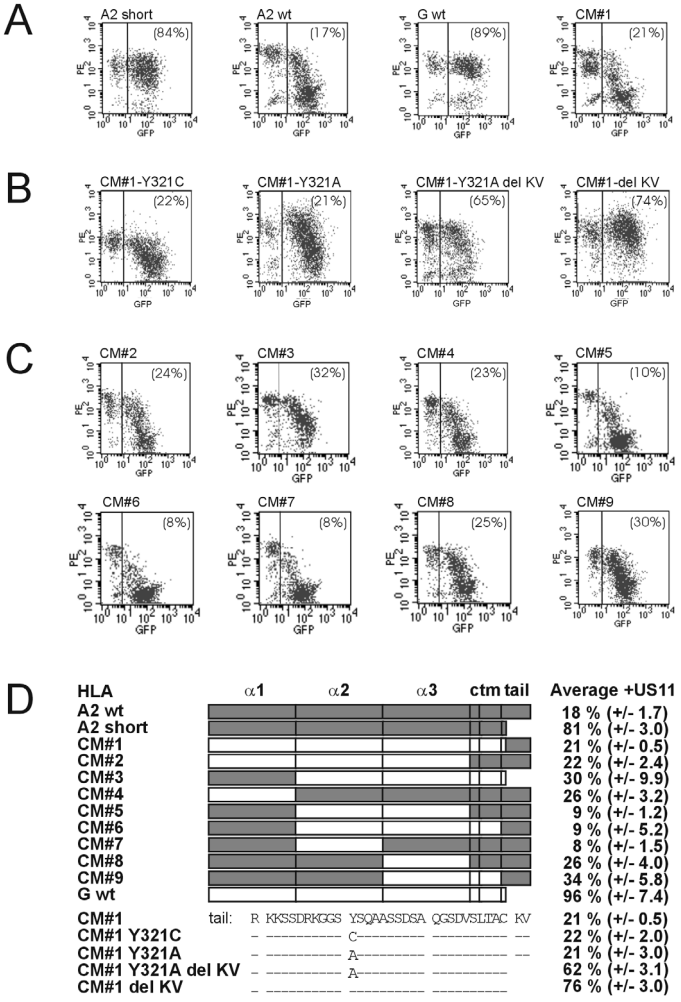

FIGURE 3. Effect of US11 on cell surface expression of wt type and chimeric MHC class I molecules. J26 cells were transfected with different HLA constructs and subsequently transduced with US11/EGFP expressing retrovirus. MHC class I molecules were labeled using W6/32, 87G (chimeras with α1 domain of 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-

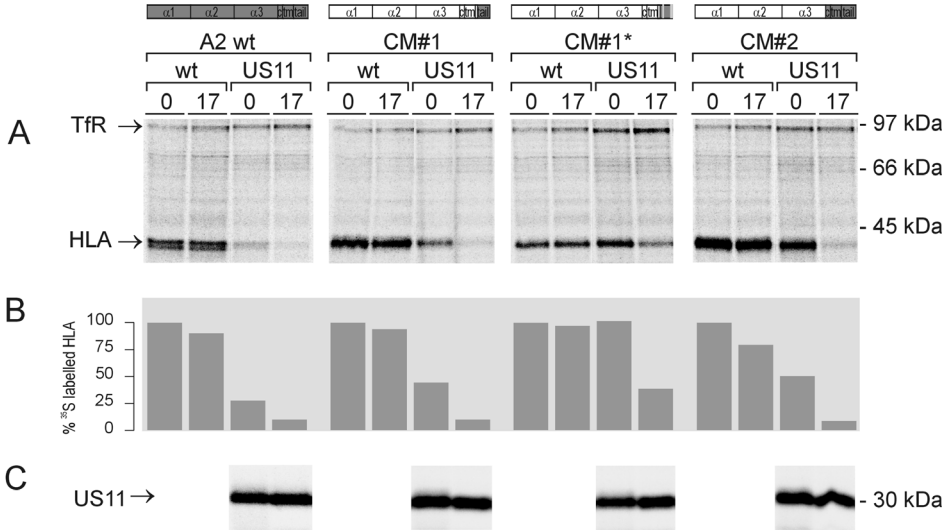

FIGURE 4. HLA-G chimeras with an extended cytoplasmic tail are susceptible to US11-mediated degradation. J26 cells transfected with HLA-A2, CM#1, CM#1 Y321A del KV (CM#1*) or CM#2 were infected with wt or US11-expressing vaccinia virus. Cells were labeled (8 min.), 4½ hours after infection, and chased 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,

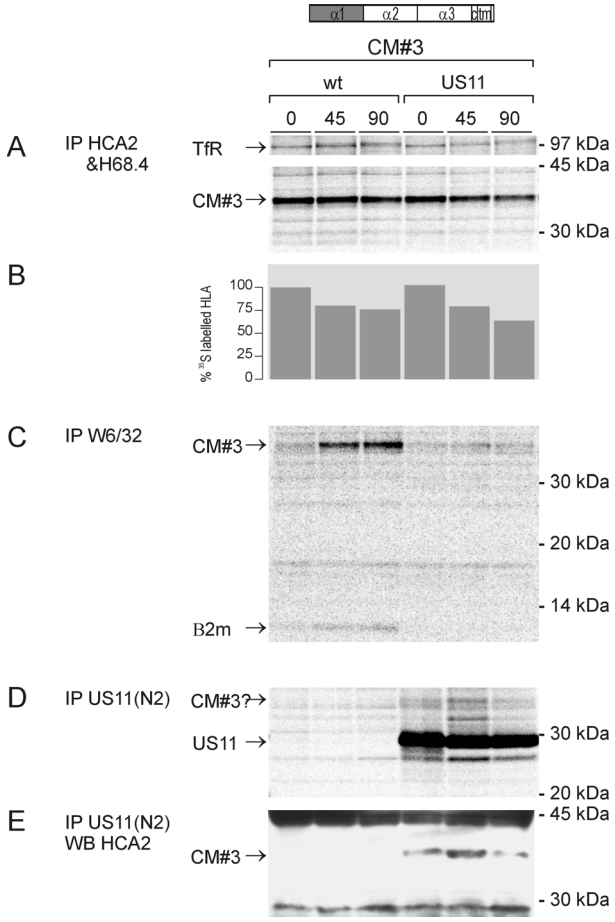

FIGURE 5. The tailless chimera of HLA-G with the α1 domain of HLA-A2 (CM#3) associates with US11 and is retained intracellularly. J26 cells transfected with HLA-CM#3 were infected with wt or US11- expressing vaccinia virus. Cells were labeled (15 min), 4 ½ hours after infection and chased for 0, 45 or 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.

Acknowledgments

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CHAPTER 3

HCMV-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane-bound, but not soluble HLA-G1 for degradation

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HCMV-encoded US2 differentially affects surface expression of MHC class I locus products and targets membrane bound, but not soluble HLA-G1 for degradation

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Human cytomegalovirus (HCMV) can elude cytotoxic T lymphocytes as well as NK cells by modulating surface expression of MHC class I molecules. This strategy would be most efficient if the virus would selectively down-regulate viral antigen- presenting alleles, while at the same time preserving other alleles to act as inhibitors of NK cell activation. We focussed on HCMV-encoded US2, which binds to newly synthesized MHC class I heavy chains and supports their dislocation to the cytosol for subsequent degradation by proteasomes. We studied the effect of US2 on surface expression of individual class I locus products using flow cytometry. Our results were combined with crystal structure data of complexed US2/HLA-A2/β2m and alignments of 948 HLA class I database sequences of the ER lumenal region inplicated in US2 binding. This study suggests that surface expression of all HLA-A, -G and most -B alleles will be affected by US2. Several HLA-B alleles and all HLA-C and -E alleles are likely to be insensitive to US2-mediated degradation. We also found that the MHC class I ER-lumenal domain alone is not sufficient for degradation by US2, as illustrated by the stability of soluble HLA-G1 in the presence of US2. Furthermore, we showed that the membrane bound HLA-G1 isoform, but also tailless HLA-A2, are targeted for degradation. This indicates that the cytoplasmic tail of the MHC class I heavy chain is not required for its dislocation to the cytosol by US2.

A large proportion of the world population is carrier of human cytomegalovirus (HCMV), which usually gives rise to asymptomatic but lifelong infections. In immuno-compromised individuals, however, HCMV contributes to high morbidity and mortality rates. Besides this, HCMV is a notorious risk factor for congenital disorders if the mother undergoes primary infection during pregnancy [1].

The host immune system is eluded by HCMV through a variety of defense mechanisms that it acquired while co-evolving with its host. Surface expression of antigen presenting MHC class I molecules, which play a central role in detection and elimination of infected cells, is affected by the concerted action of a set of HCMV unique short (US) region- encoded proteins (US2, US3, US6, US11) expressed along different stages of viral infection [2-6]. Like US11, US2 induces proteolytic degradation of MHC class I molecules: immediately after their synthesis and translocation into the ER lumen, MHC 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;6;7]. When devoid of MHC class I surface expression, cells may become a target for NK cell lysis [8]. Several mechanisms have been described by which HCMV can sidetrack NK cells, either by blocking expression of ligands that activate NK cells or allowing expression of ligands that inhibit NK cell triggering. Several proteins encoded within the UL region of the HCMV genome (UL16, UL18, UL40) could protect from NK cell lysis [9]. In addition, inhibitory signals to NK cells could be preserved by allowing selective surface expression of those MHC class I that are less efficient in viral antigen presentation [10].

Most host cells express HLA-A, -B, -C and -E alleles. HLA-A and –B alleles are likely most powerful in the presentation of viral antigens, which is supported by a very high degree of polymorphism in the world population for these alleles [11]. The contribution of HLA-C to immune control by cytotoxic T cells is not entirely clear. HLA-C alleles are less polymorphic and surface expression is generally lower then that observed for HLA-A and -B alleles, but HIV peptides displayed in the context of HLA-C have been reported [12;13]. HLA-E is low polymorphic, mainly presents peptides derived from signal sequences of MHC class I molecules, and is expressed in low amounts at the cell surface [10]. HLA-E has also been reported to present peptides to specific T cell receptors on CD8+ T cells or NK CD8+ cells [14] [15]. HLA-E and HLA-C could also serve important functions as inhibitory NK cell receptor ligands [16;17].

HLA-G is found primarily on extravillous cytotrophoblast cells at the fetal-maternal interface and on a subpopulation of thymic epithelial cells [18]. These trophoblast cells express no HLA-A and -B alleles on their cell surface, only HLA-C, -E and -G. Different HLA-G isoforms have been described, of which only one, called HLA-G1, is expressed at the cell surface [19]. HLA-G1 has a unique feature: it has a relatively short cytoplasmic tail of 6 amino acids. Another isoform, soluble HLA-G1, has all domains required for stable complex formation with β2m and peptide and is secreted [20]. Membrane bound and soluble HLA-G both present viral peptides to CD8+ T cells [21-23]. The soluble isoform also induces apoptosis of activated CD8+ T cells and inhibits CD4+ T cell proliferation [24-26]. It is unknown to what extent this potential T cell function is exploited, because only few macrophages and T and B immune cells are found at the implantation site. The population of lymphoid cells at this site predominantly consists of NK cells [27]. Like other HLA class I molecules, HLA-G could also be an important modulator of cytokine production by NK cells. Interactions with activating NK receptors and stimulation of cytokine secretion by uterine NK cells could play a role in the placentation process. HLA-G could also serve as ligand for inhibitory NK receptors to prevent trophoblast lysis [27].

For efficient immune escape, HCMV should prevent the display of viral antigens by MHC class I molecules to cytotoxic T cells and at the same time preserve a substantial amount of ligands that can modulate NK cell activation. It would be beneficial to selectively down-regulate those MHC class I molecules that are most important for viral antigen presentation and keep other locus products as inhibitor of NK cell activation.

In general, HLA-A and -B alleles are believed to be down-regulated by HCMV, but detailed information is scarce. Pulse chase experiments suggested that US2 does not affect the stability of HLA-C, -G, and -E alleles [28;29].

In the present study, we evaluated the sensitivity of different MHC class I locus products to US2-mediated down-regulation using flow cytometry. Flow cytometrical analysis of MHC class I surface expression is most relevant with respect to T and NK cell interactions. Besides this, it can be a valuable complementation of pulse chase data as it could unravel or exclude other mechanisms than degradation that cause reduced surface expression.

We introduced different HLA class I alleles into a murine cell line, which also expresses human β2m, and monitored the effect of US2 on surface expression of these molecules. To verify that our results were not species or cell type specific, we included experiments with human cell lines. We further investigated structural requirements for US2-induced HLA class I heavy chain degradation, making use of membrane-bound HLA-G1, which lacks most cytoplasmic tail residues, and soluble HLA-G1, which consists of ER-lumenal domains only. Or data will also show that US2 and US11employ different strategies to target class I heavy chains for degradation.

MATERIALS AND METHODS

Cell lines

J26 cells (H-2^k murine Ltk$^-$ cells expressing human β2m) [30], the Phoenix amphotropic retroviral producer cell line and JEG-3 cells (both from the American Type Culture Collection, ATCC) were cultured in DMEM (Life Technologies Inc). J26 cells expressing HLA-B7 (B*070201), B27 (B*270502) and Cw3 (Cw*030401) were described previously [31;32]. U373-MG cells (ATCC) were cultured in RPMI 1640 medium (Life Technologies Inc.). All media were supplemented with 10% FCS (Greiner), 100 U/ml penicillin and 100 μg/ml streptomycin and G418 (GibcoBRL).

Antibodies

The following anti-MHC class I mAbs were used for flow cytometry: 87G (HLA-G) [23], W6/32 (general HLA class I) [33], MA2.1 (HLA-A2) [34], MEM-E/06 (HLA-E; EXBIO Praha, Czech Republic), B1.23.2 (HLA-C)[35]and Y-3 (murine MHC class I; ATCC). PE-conjugated goat anti-mouse (gαm), IgG (Jackson Immuno Research laboratories) was used as second Ab. The polyclonal antisera MR24 (directed against the α3 domain of HLA-A*0201 and generated in rabbits using synthetic tetanus toxoid conjugated peptide PKTHMTHHAVSDHEA) and US2-N2 (directed against the N-terminus of US2 and generated in rabbits using synthetic tetanus toxoid conjugated peptide GITKAGEDALRPWKSTAK), as well as mAbs HCA2 (HLA-A, -G) [36], MEM-G/01 (HLA-G; EXBIO Praha, Czech Republic) and H68.4 (transferrin receptor; Zymed), were used for immunoprecipitations.

Construction of plasmids

Plasmid pLUMC9901 (encoding HLA-A*0201) [37], was used as template for the construction of short-tailed

HLA-A2 constructs containing 4 or 6 amino acid tails (-RRKS or -RRKSSD respectively). A cDNA fragment obtained from an HLA-Cw4 typed individual was used for subcloning into pcDNA3.1/V5/His-TOPO to generate the plasmid pCw*0304 (generous gift of B. van den Eynde). The HLA-Cw4 fragment was fully sequenced to determine the HLA-Cw*0304 allelic subtype. Plasmid pCw*0304 and pLUMC9901 were used for the construction of HLA-Cw3/HLA-A2 chimeras. For HLA-Cw3/tail A2, the HLA-Cw3 cytoplasmic tail was replaced with the corresponding HLA-A2 tail region (aa 310-342). The reverse was done for the construction of HLA-A2/tail Cw3. These constructs were generated applying the megaprimer method [38] and were subcloned into pcDNA3 (Invitrogen). Plasmid pcHLA-A/E was generated by replacing the leader sequence of the HLA-E*01033-Gly^{107} gene derived from cosmid 3.14 [39] with the leader sequence of HLA-A2 and subcloning this into pcDNA3. Plasmid pcDNA-G1 (encoding HLA-G*01011) and soluble HLA-G1 cDNA have been described previously [40;41]. Amplifications were performed using DNA polymerase *Pwo* (Eurogentec, Seraing, Belgium). All constructs were fully sequenced to verify the absence of unwanted mutations.

Transfection

J26 and U373 cells were transfected with the different MHC class I constructs using Effectene™ Transfection Reagent (Qiagen). After 48 hours, stable transfectants were selected by adding 0.3 mg/ml G418 (GibcoBRL). Cells were sorted by flow cytometry for expression of the introduced cDNA as described [40].

Production of retrovirus and transduction

A retroviral vector expressing both US2 and EGFP was constructed by ligating a US2-encoding cDNA fragment into the pLZRS-*IRES*-EGFP vector [42]. Wild type or US2-*IRES*-EGFP constructs were transfected into the amphotropic Phoenix packaging cell line for the production of retrovirus, as described [40]. Cells were transduced with retrovirus using retronectin (Takara, Japan) coated dishes.

Vaccinia virus infections

Cells were infected with wild type or US2-expressing recombinant vaccinia virus (generous gift of Dr. J.Yewdell) at a multiplicity of infection of 10 PFU/cell for 45-60 min in a small volume (~1.5 ml) of serum free culture medium at 37°C [43]. After infection, a mix of conditioned and fresh culture medium was added. Metabolic labeling of the infected cells was performed approximately 4½ h after infection.

Flow cytometry

Cell surface expression of MHC class I molecules as well as EGFP expression in cells transduced with retrovirus were analyzed using flow cytometry as described [40]. J26-HLA-E and JEG-3 cells were stained in 3 steps to intensify the MHC class I staining: first with specific anti-MHC class I antibody, then with biotinylated-gαm and finally with streptavidin-conjugated PE. Absence of MEM-E/06 binding to wild type J26 cells in flow cytometry (data not shown) excluded any possible cross reactivity with murine MHC class I. Data are collected from at least two independent experiments of which one representative experiment is shown. Independent measurements for MHC class I (PE) staining in EGFP positive cells differed with an average of 5%.

Metabolic labeling, immunoprecipitation and SDS-PAGE

Metabolic labeling, immunoprecipitations and SDS-PAGE were performed as described [37]. 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 proteasome inhibitor ZL_3H. Cells were lysed in a small volume of Nonidet-P40 lysis buffer, containing protease inhibitors leupeptin, AEBSF and ZL_3H, for 30 min at 4°C After centrifugation to remove cell debris, supernatant was transferred to a new tube to which 1/10 volume of 10% SDS and 1/10 volume of 0.1 M DTT was added. Samples were boiled for 5 min to further denature proteins. Next, the volume was increased 10 times with non-denaturing buffer (1% Triton X-100, 50 mM Tris HCl pH 7.4, 300 mM EDTA, 0.02%NaN_3) supplemented with protease inhibitors (leupeptin, AEBSF, ZL_3H) and 10 mM iodoacetic acid. Immunoprecipitations were performed ≥ 2 h on precleared samples, with Abs pre-coupled to protein A/G sepharose beads. Samples were separated by SDS-PAGE and displayed via phosphor imaging (Bio-Rad Personal Molecular Imager FX).

RESULTS

Allelic differences in US2-mediated down-regulation of MHC class I surface expression.

We first investigated if US2 differentially affects surface expression of various MHC class I alleles.

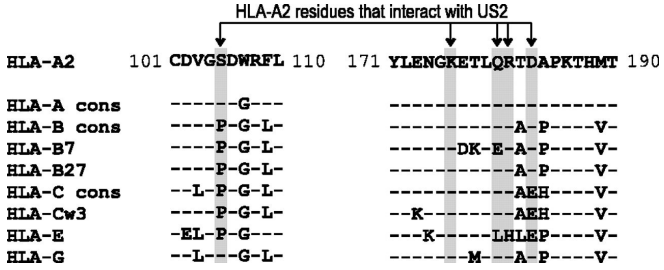

FIGURE 1. Overview of sequence variation within the US2 binding region of MHC class I locus products. Depicted is the region of HLA-A2 with residues directly involved in interaction with US2 marked in grey (according to crystal structure data from PUBMED Protein Data Base reference: 1IM3, deposited by Gewurz *et al.*[44]. The US2 binding site of HLA-A2 has been aligned with corresponding regions of other alleles, and their locus (sub)group consensus sequences (cons) obtained from the IMGT/HLA Sequence Database [45].

Since alterations in surface display of MHC class I can directly affect the incidence of T and NK cell receptor interactions, we used flow cytometry for our investigation. The available crystal structure data on a soluble complex of US2/HLA-A2/β2m provided a starting point for the selection of representative locus products for our study [44]. By aligning HLA class I sequences from the IMGT/HLA sequence database [45] to the region of US2-interaction described for HLA-A2 and looking at conserved residues, we found HLA-A2, -B7, -B27, -Cw3, -G and -E alleles to be good candidates (see Figure 1). These alleles all have an amino acid composition for the region implicated in US2 binding that is conserved within their locus (sub)group. HLA class I constructs were transfected into murine J26 cells to enable specific monitoring of introduced alleles by flow cytometry. J26 cells co-express human β2m to allow proper HLA class I complex formation. Cells were then transduced with retrovirus expressing US2-*IRES*-EGFP. Figure 2 shows that surface expression of HLA-A2, -B27 and -G was reduced by US2, to 12%, 39% and 7% respectively, compared to US2-negative cells. In contrast, HLA-B7, -Cw3 and -E are not or only slightly affected by US2 with 110%, 85% and 98% surface expression, respectively. Endogenously expressed H-2^k molecules were not downmodulated by US2. Control EGFP-expressing retrovirus had no effect on MHC class I cell surface expression, as shown here for HLA-A2.

Cytoplasmic tail of MHC class I heavy chains is not essential for US2-mediated down-regulation

We previously showed that residues at the extreme end of the cytoplasmic tail of class I heavy chains can

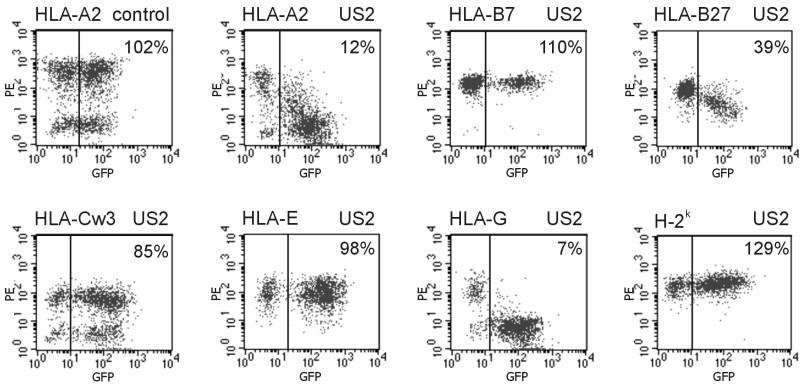

FIGURE 2. Selective US2-mediated down-regulation of HLA class I. Murine J26 cells, transfected with different plasmids encoding HLA class I and transduced with US2-*IRES*-EGFP encoding retrovirus, were analyzed using flow cytometry. The following MoAbs were used to stain the different HLA class I molecules: HLA-A2 (MA2.1), HLA-B7, -B27, and -Cw3 (W6/32), HLA-E (MEM-E/06), HLA-G (87G) or endogenously expressed H-2^k (Y-3), followed by PE-conjugated goat anti-mouse Ab (Y-axis). US2 positive cells are marked by EGFP expression (X-axis). The effect of US2 on surface staining of MHC class I was calculated by comparing mean PE fluorescence of EGFP negative (defined as 100%) and positive cells. As a control, J26-HLA-A2 cells were transduced with EGFP-expressing retrovirus to show that EGFP alone, does not affect MHC class I surface expression.

determine their sensitivity to down-regulation by US11 [40]. Others reported that a reduction of the tail to 4 amino acids ensures stability of HLA-A2 in the presence of either US11 or US2 [46]. HLA-G has a relatively short cytoplasmic tail of 6 amino acids [47], due to a premature stopcodon in exon 6, and was found to be insensitive to US2-mediated degradation [28]. We decided to evaluate the contribution of the tail of class I heavy chains to US2-mediated down-regulation in more detail (Figure 3). By exchanging cytoplasmic tail regions of US2-sensitive (HLA-A2) and US2-insensitive (HLA-Cw3) class I molecules (see Figure 3A), we evaluated if the tail region could account for differences in sensitivity among locus products. Figure 3B shows that HLA-A2 with the tail of HLA-Cw3 was down-regulated as efficiently as HLA-A2 wt (both 12% surface expression in the presence of US2), while HLA-Cw3 with the tail of HLA-A2 and wild type HLA-Cw3 were almost equally resistant (with 75% and 85% surface expression). In parallel, we evaluated the effect of tail length on US2-sensitivity by reducing the size of the tail of HLA-A2 molecules (see Figure 3A) to 6 amino acids (similar in length to HLA-G) or 4 amino acids (identical to the construct tested previously [46]). Figure 3B shows that both short-tailed HLA-A2 molecules were efficiently downmodulated by US2.

These data indicate that the tail of MHC class I heavy chains is not essential for US2-mediated down-regulation of cell surface expression.

HLA-G and short-tailed HLA-A2 are efficiently targeted for degradation by US2.

Downregulation of MHC class I surface expression can be accomplished by degradation, but also by retention in an intracellular compartment, as has been shown for US11 [40]. We investigated the underlying mechanism for down-regulation of short-tailed MHC class I molecules by US2. Since the tail region of the class I heavy chain is exposed to the cytosol it might serve an important function in the retrograde transport to the cytosol for subsequent proteasomal degradation. We investigated whether US2 affects the stability of HLA-G1 and short-tailed HLA-A2 (6 aa tail) in a pulse chase experiment (Figure 4). Recombinant vaccinia virus was used to introduce US2 into the cells. In parallel, a similar amount of cells was infected with wild type vaccinia virus. Immunoprecipitations were performed on denatured cell lysates to evaluate the effect of US2 on total amounts of HLA class I heavy chains, irrespective of their folding state.

We observed a destabilizing effect of US2 on wt HLA-A2 as well as on HLA-G1 and short-tailed HLA-A2 (upper panel Figure 4). In the presence of proteasome inhibitor ZL_3H (lower panel) deglycosylated degradation intermediates could be observed in US2 expressing cells for all three HLA class I molecules.

Thus, HLA-G1 and short-tailed HLA-A2 are efficiently destabilized in $US2^+$ cells, indicating that the cytosolic tail of MHC class I heavy chains is not required for US2-dependent dislocation and degradation.

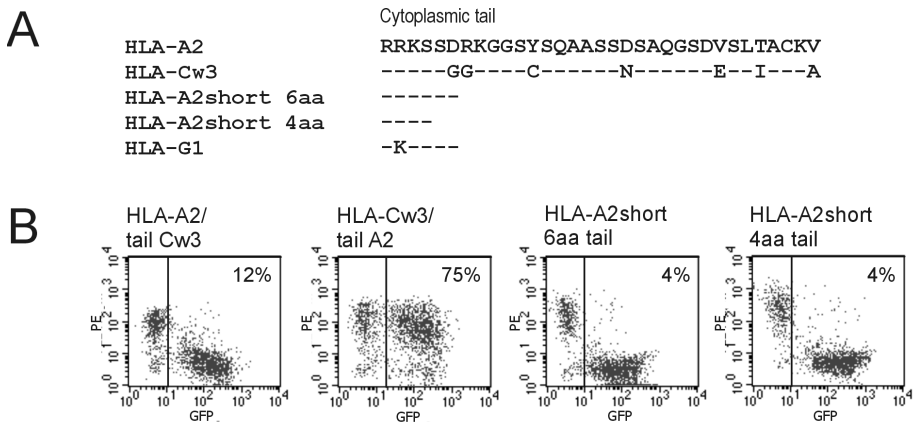

FIGURE 3. Cytoplasmic tail of MHC class I is not essential for US2-mediated down-regulation. A) Overview of amino acid sequences of the cytoplasmic tail regions that are exchanged in the HLA-A2/Cw3 chimeras and shortened for the HLA-A2 tail mutants. As a comparison, the HLA-G tail region is depicted. **B)** J26 cells were transfected with chimeras or tail mutants, transduced with US2-*IRES*-EGFP encoding retrovirus and analyzed using flow cytometry. MHC class I molecules are stained with W6/32 (chimeras) or MA2.1 (HLA-A2short), followed by goat anti-mouse-PE conjugate (Y-axis). US2 positive cells are marked by EGFP expression (X-axis).

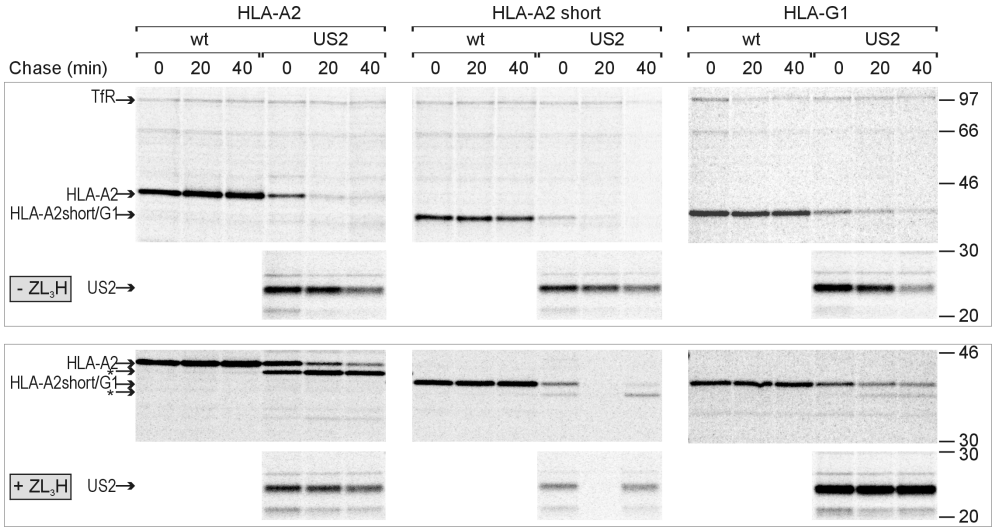

FIGURE 4. HLA-G and short-tailed HLA-A2 are efficiently degraded in US2 expressing cells. J26 cells expressing wt HLA-A2, HLA-A2short (6aa tail) or HLA-G were infected with wt or US2-encoding vaccinia virus. At 4½ hours post-infection, cells were metabolically labeled for 10 minutes and chased for 0, 20 or 40 minutes in the absence (upper panel) or presence (lower panel) of proteasome inhibitor ZL_3H. Immunoprecipitations were performed on denatured cell lysates using the following antisera: MR24 (HLA-A2 wt, -A2short), HCA2 (HLA-G), H68.4 (transferrin receptor [TfR]) or US2-N2 (US2) Abs. Deglycosylated degradation intermediates are marked by an asterisk. The 20 min chase sample (+ZL_3H, +US2) for HLA-A2short was lost during the procedure for this representative experiment. Molecular weight markers are given in kilo Daltons.

Evaluation of US2-mediated down-regulation of HLA-G in cell lines of human origin.

To ensure that our observation on HLA-G was not species or cell type specific, we included experiments in different cell lines of human origin (Figure 5).

First, U373 astrocytoma cells (expressing HLA-A2, -B18 and -Cw5), transfected with HLA-G and transduced with control or US2-*IRES*-EGFP encoding retrovirus, were analyzed by flow cytometry (Figure 5A). Comparable HLA-G surface expression was observed in control EGFP expressing cells and non-transduced cells, as determined with 87G (upper left histogram). This antibody is specific for HLA-G and does not cross-react with endogenous HLA class I, as wild type U373 cells remained PE negative when stained with this antibody (Figure 5A, lower left histogram). A reduction of HLA-G surface expression was seen in US2/EGFP expressing cells (to 45%). Down-regulation of HLA-G by US2 seems to be even more efficient than that of endogenous HLA-A2, stained by antibody MA2.1 (to 63%). This antibody preferentially recognizes HLA-A2 molecules and not HLA-B18 or -Cw5 in these U373 cells [34]. MA2.1 did not cross-react with HLA-G in HLA-G1 expressing J26 cells (data not shown).

Note that wild type U373 cells expressing US2 demonstrated a stronger down-regulation of endogenous HLA-A2 molecules stained with MA2.1 (to 15%). This indicates that the transfection of an additional US2-sensitive class I heavy chain into cells affects the efficiency of down-modulation of the total pool of MHC class I. It is unlikely that the lower efficiency of MHC class I down-regulation in the U373-HLA-G cells is due to a lower level of US2 expression. The mean fluorescence value for EGFP, as a marker for US2 expression (not shown in figure 5A) was even higher in these cells (255) than in wild type U373 cells (194).

Secondly we tested the effect of US2 on HLA-G in the physiologically more relevant trophoblast cell line JEG-3 (Figure 5B). These cells naturally express HLA-G1, HLA-Cw4 and likely also low amounts of HLA-E, but lack HLA-A and HLA-B molecules at their cell surface. In cells transduced with US2 retrovirus, HLA-G surface expression was severely reduced (to 9%) whereas HLA-Cw4 surface expression remained stable. In this case, severity of HLA-G down-modulation was comparable to that observed for J26 cells transfected with HLA-G (to 7%, see Figure 2).

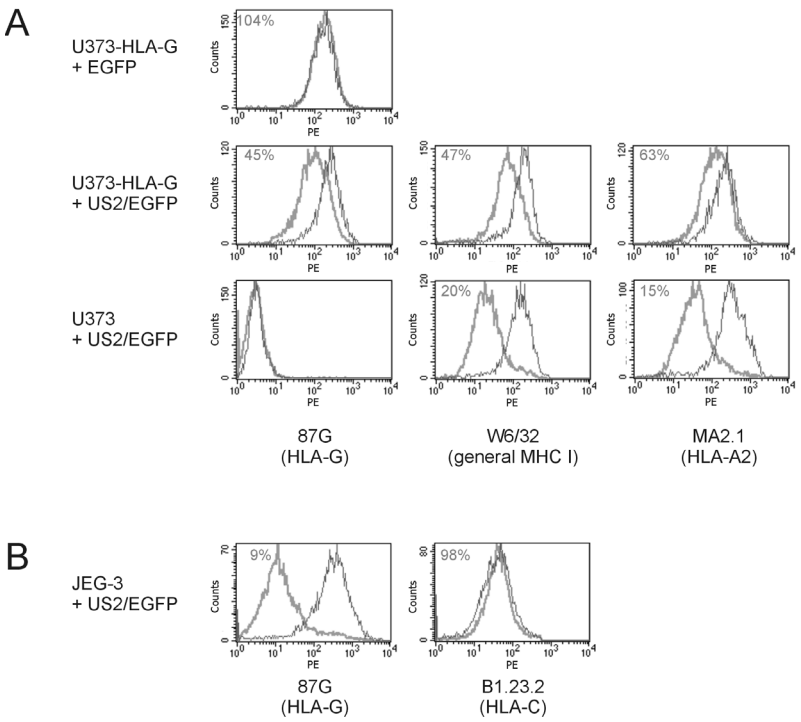

FIGURE 5. US2-mediated down-regulation of HLA-G in human cell lines. A) Wild type human U373 astrocytoma cells (HLA-A2, -B18, -Cw5), or U373 cells transfected with HLA-G were transduced with retrovirus (EGFP- or US2-*IRES*-EGFP) and analyzed by flow cytometry. Cells were stained with either 87G (HLA-G), MA2.1 (HLA-A2), or W6/32 (general anti-MHC class I) MoAbs, followed by goat anti-mouse PE conjugate. Shown are histograms representing transduced (thick grey line) and non-transduced (thin black line) cells. Surface expression is given as percentage of mean PE fluorescence in transduced cells compared to non-transduced cells. **B)** Similarly, JEG-3 cells expressing HLA-G1, -Cw4 (and likely also HLA-E) were transduced with retrovirus (US2-*IRES*-EGFP), stained with 87G (anti-HLA-G) or B1.23.2 (anti-HLA-C) and analyzed using flow cytometry.

Note that both cell lines expressed no other US2-sensitive MHC class I molecules besides HLA-G.

Together, these results show that down-regulation of short-tailed MHC class I molecules can be observed in different cell lines of human origin. These data further indicate that efficiency of down-regulation is influenced by amounts of US2-susceptible class I heavy chains present in the cells.

Soluble HLA-G1 is resistant to US2-mediated degradation

Interaction between US2 and class I heavy chains does not require transmembrane regions, as soluble trimeric HLA-A2/US2/β2m complexes could be crystallized [44]. Binding of MHC class I molecules to a soluble form of US2 is not sufficient for their degradation [48]. We investigated if interaction of US2 with a soluble form of MHC class I could induce its degradation. This is of clinical relevance, as membrane-bound and soluble isoforms are generated by differential splicing of primary HLA-G mRNA transcripts [20]. One of these isoforms is soluble due to retention of intron 4, which introduces a premature stopcodon (Figure 6A). This isoform possesses the ER-lumenal α1-3 domains required for interaction with US2. We compared its sensitivity to US2-mediated degradation to that of the membrane bound HLA-G isoform in pulse chase experiments (Figure 6B). In the absence of proteasome inhibitor, a progressive loss of membrane bound HLA-G1 and endogenous MHC class I molecules was observed in US2-expressing cells (upper panel). In contrast, soluble HLA-G1 remained stable throughout the chase (lower panel). In the presence of proteasome inhibitor, a deglycosylated degradation intermediate could be observed for both endogenous HLA molecules and membrane bound HLA-G1, but not for soluble HLA-G1.

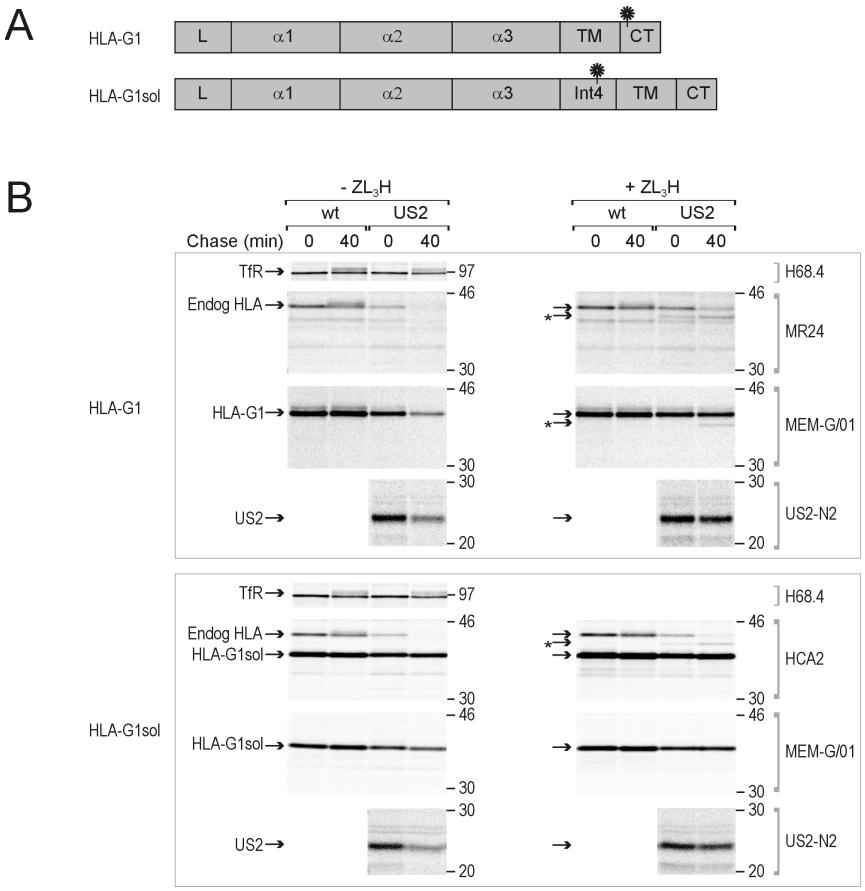

FIGURE 6. Soluble HLA-G1 is stable in US2-expressing cells. A) Depicted are two isoforms of HLA-G: membrane bound and soluble HLA-G1. HLA-G1 has a premature stopcodon (*) in exon 6 which allows translation of only 6 amino acids of the cytoplasmic tail (CT) region. Soluble HLA-G1 contains a premature stopcodon due to retention of intron 4, allowing translation of only ER-lumenal α1-3 domains. L=leader sequence, TM=transmembrane region **B)** U373 cells transfected with membrane bound or soluble HLA-G1 were infected with wt or US2-encoding vaccinia virus. At 4½ hours post-infection, cells were labeled for 10 minutes and chased for 0 or 40 minutes in the absence or presence of proteasome inhibitor ZL_3H. Immuno-precipitations were performed on denatured cell lysates with MR24 (endogenous HLA class I), MEM G/1 (HLA-G), H68.4 (TfR) or US2-N2 (US2) Abs. Deglycosylated degradation intermediates are marked by an asterisk.

Evidently, soluble HLA-G1 can escape US2-mediated degradation although it possesses all ER-lumenal α1-3 domains required for binding US2.

DISCUSSION

HCMV encodes several proteins that interfere with cross talk between host cells and immune effector cells through modulation of surface expression of MHC class I molecules. The various MHC class I locus products can serve different immune functions. Some are more important for the presentation of viral antigens, while others may mainly act as ligands for inhibitory NK receptors. The success of immune escape by HCMV through modulation of MHC class I surface expression is likely to be influenced by MHC class I allele specificity of the different HCMV US proteins. In this paper we focused on modulation of MHC class I expression by HMCV US2.

We first evaluated allelic differences in US2-mediated down-regulation of MHC class I cell surface expression. Previous studies on US2 mainly focused on the mechanism of interference with antigen presentation [2;7;46;48;49]. With the cell lines and

antibodies used in these studies it is difficult to deduce effects on individual MHC class I locus products. In general, HLA-A and -B alleles are believed to be down-regulated, but detailed information is scarce. Binding studies indicate that US2 associates with HLA-A2 and -Aw68, but no interaction could be detected with HLA-B7, -B27, -Cw4, or -E [50]. Pulse chase experiments show that US2 does not affect the stability of HLA-C, -G, and -E alleles [28;29]. It is, however, also important to specifically evaluate the effect on surface expression of MHC class I molecules, as this is most relevant with respect to T and NK cell interactions. MHC class I molecules that appear to be stable in pulse chase experiments can nevertheless be withheld from the cell surface via other mechanisms than degradation. US11, for example, can cause ER-retention of MHC class I molecules that can not be targeted for degradation [40]. Flow cytometrical analysis of surface expression therefore is a valuable complementation of pulse chase data.

The available crystal structure of a soluble complex of US2/HLA-A2/β2m [44] provided us with a good starting point for the selection of representative class I locus products (Figure 1). We found that US2 down-regulates HLA-A2, -B27, and -G, but not HLA -B7, -Cw3, and -E alleles of this selection (Figure 2). Sequence variation in the region implicated in US2 binding (residues 101-110 and 171-190) was evaluated for those alleles present in the IMGT/HLA sequence database [45] that are fully sequenced for this region. Of the residues directly involved in interaction with US2, the residue at position 105 is either S or P, and both are found in US2 sensitive alleles. Out of 947 alleles, 946 have K176. Residues at position 105 and 176 are therefore unlikely to account for sensitivity differences between locus products. Among locus products that differ in sensitivity for US2, residues found at positions 180, 181 or 183 also differ. All 274 HLA-A and most of the 519 HLA-B alleles have residues Q180, R181 and D183 that are present in US2-sensitive HLA-A2 and HLA-B27. In contrast, US2-insensitive HLA-B7 has E180 instead of Q180, which is also found in other HLA-B alleles. Two other US2-insensitive locus products, HLA-Cw3 and HLA-E, have E183 instead of D183. This E183 is also found in 132 out of 133 HLA-C alleles and in all 6 HLA-E alleles. In addition, HLA-E alleles differ from US2-sensitive alleles at position 180 and 181, with their L180 and H181 residues. Among the residues that are not directly involved in US2 binding, but that are located around US2 binding residues, only little sequence variation is found. Moreover, most of the sequence variation that is found here is unlikely to account for allelic differences, as they are found in both US2-sensitive and -insensitive alleles. Altogether, our findings suggest that US2 down-regulates all HLA-A, -G and most -B alleles and no HLA-C, or-E. In addition to HLA-B7 several other HLA-B isotypes, including HLA -B8, -B40, -B41, -B42, and -B48 are likely to be US2-resistant. These HLA-B isotypes are relatively common, as they are found in 25-30% of the Caucasion population (Frans Claas, personal communication). Since HLA-B alleles are very important for presentation of viral peptides to cytotoxic T cells, it might be advantageous for the host to possess such HLA-B alleles.

The finding that US2 down-regulates cell surface expression of HLA-G, which has a relatively short tail, is novel. A previous report claimed that HLA-G remains stable in the presence of US2 [28]. Resistance to US2-mediated down-regulation has been ascribed to absence of a full-length tail, as tailless HLA-A2 molecules were reported insensitive to US2-mediated degradation [46]. When looking at the residues required for interaction with US2, the HLA-G locus product closely resembles HLA-A2 (see Figure 1A). The most striking difference between HLA-G and -A locus products is found in their cytosolic domains, as HLA-G alleles lack most cytosolic tail residues due to a premature stop codon in exon 6. Our flow cytometry experiments with murine cells showed that both HLA-G1 and tailless HLA-A2 molecules were withheld from the cell surface by US2 (Figures 2 and 3). When we investigated the mechanism underlying this reduction in cell surface expression, it became evident that HLA-G1 and tailless HLA-A2 molecules are targeted for degradation by US2 (Figure 4). These data reveal that the cytosolic tail is not required for US2-mediated dislocation and subsequent degradation. To exclude that cell- or species-specific factors account for our observations in the murine cell line, we also performed experiments in human JEG-3 trophoblast and U373 cell lines, with similar results (Figures 5 and 6). The observation that tailless HLA class I molecules are sensitive to US2-mediated degradation may have been missed in earlier reports as a result of a different experimental set up; The experiments shown by Schust et al. [28] are performed with porcine endothelial cells transfected with HLA-G. However, HLA-G expression in these cells was too poor to allow firm conclusions on possible stability changes as a result of US2 expression [28]. Furthermore, our experiments with U373 cells (the same cell line used by Story et al.

[46]) indicate that expression of multiple, US2-sensitive class I products results in reduced efficiency of US2-mediated MHC class I down-regulation (Fig. 5). Down-regulation of HLA-G was more pronounced in murine J26 cells transfected with HLA-G or in naturally HLA-G expressing JEG-3 trophoblast cells. In contrast to the U373 cell line transfected with HLA-G, these cell lines express no other US2-sensitive MHC class I molecules than HLA-G. The effectiveness of down-modulation of HLA products likely also depends on expression levels of US2, which may vary between expression systems. US2-mediated degradation of tailless HLA-A2 molecules might have been revealed in previous experiments [46] if the experiments had been performed in the presence of proteasome inhibitor. In case of a suboptimal degradation efficiency, a small amount of deglycosylated degradation intermediate already serves as indisputable evidence.

We also investigated if US2 could induce degradation of the soluble HLA-G1 isoform. This isoform consists of ER-lumenal α1-3 domains only. It is known that interaction between US2 and HLA class I does not require transmembrane regions, as soluble US2/HLA-A2/β2m complexes could be crystallized [44]. We have shown here that ER-lumenal domains of HLA class I alone are nevertheless insufficient to allow US2-mediated degradation, as soluble HLA-G1 remained stable in the presence of US2 (Figure 6).

The observation that viruses use different proteins for interference with MHC class I expression is likely to improve the effectiveness of immune escape. Together they can affect a wider range of class I locus products. They may also act synergistically to reach a more dramatic down-regulation of a single locus product. The use of different strategies may also help to avoid saturation of a particular cellular pathway used by the virus to effectuate evasion. HCMV encodes several proteins that affect MHC class I surface expression during the course of infection, and they act through different mechanisms: a) by retaining MHC class I molecules in the ER (US3) [3], b) by blocking the Transporter associated with Antigen Processing (TAP)(US6) [4;5] c) by delaying MHC class I maturation (US10) [51], and d) by dislocating newly synthesized class I heavy chains to the cytosol for subsequent degradation by proteasomes (US2 and US11) [2;6]. When looking at the different mechanisms employed, US2 and US11 seem to act very similarly. Our data contribute to the understanding of the need of two proteins with, at first sight, similar functions. Previous studies have already shown that US2 and US11 each interact with different sites on ER-lumenal regions of the HLA class I heavy chain [40;44]. We showed here that they also differ in their requirements for cytosolic tail residues, as HLA-G1 and tailless HLA-A2 are efficiently targeted for degradation by US2. As shown before, this is not the case for US11 [28;40;46]. Interestingly, the requirement for cytosolic tail residues appears to be the reverse for the viral proteins themselves. Where tailless US11 can still target newly synthesized class I heavy chains for degradation, this is not the case for US2 [48;52]. Since US2 and US11 approach their target MHC class I molecules differently, they may affect different subsets of locus products. The difference in cytosolic tail requirements may also be accompanied by usage of different accessory proteins involved in the dislocation and degradation process.

Trophoblast membrane-bound and soluble HLA-G1 are believed to serve important immunological functions at the fetal-maternal interface. Our finding that membrane-bound, but not soluble HLA-G1, is sensitive to US2-mediated degradation may be of relevance in the context of HCMV infection during pregnancy.

It is unknown if viral antigen presentation could be effective at the fetal-maternal interface. Only few maternal CTLs are found here and during HCMV infection, T cell recognition of infected trophoblast cells will largely be prohibited due to absence or reduction of surface expression of antigen presenting MHC class I molecules: HLA-A and -B molecules are lacking in these cells and surface expression of HLA-G is reduced by both US2 (this paper), US3 [53] and US6 [54]. In addition, HLA-C can be down-regulated by US3, US6 [53] and US11 [55] ,(our own unpublished results). HLA-E surface expression is not affected by US2 (this paper), US6 [54] and US11 (our own unpublished results). We are unaware of data describing the effect of US3 on HLA-E expression.

NK cells are the predominant population of immune effector cells at the maternal-fetal interface and they serve several important functions at this site. By down-regulating most HLA class I locus products, many ligands for inhibitory receptors on NK cells will also be lost. Expression of HLA-E and UL16, UL18 and UL40 is not likely to fully compensate for the lack of inhibitory NK cell ligands [8]. It is unclear if HCMV-infected trophoblast cells are at risk for NK cell attack *in vivo*, as the degree of protection from NK cell lysis

was shown to differ significantly between lab strains and clinical isolates [56]. HLA-G down-regulation by HCMV could also prevent interaction with activating NK cell receptors and prohibit uterine NK cell cytokine secretion, which is necessary for the placentation process.

We also demonstrated that soluble HLA-G1 is resistant to US2-mediated degradation. In a previous paper we showed that US11 has no effect on surface expression of membrane-bound HLA-G1 [40], which makes it unlikely that US11 will prohibit secretion of the soluble HLA-G1 isoform. It remains to be established to what extent other US proteins could affect its secretion. Soluble HLA-G1 can induce apoptosis of activated T cells [24]. Cells secreting this HLA-G1 isoform could also modulate NK cell triggering.

Together, our results suggest that surface expression of all HLA-A, and -G, and most -B alleles will be down-regulated in the presence of US2. Several HLA-B alleles and all HLA-C and -E alleles will be resistant against US2-mediated degradation. Using HLA-G and tailless HLA-A2 we demonstrated that US2 does not require the cytosolic tail of class I heavy chains to initiate dislocation and degradation of these molecules. The MHC class I ER-lumenal domain alone is not sufficient for US2-mediated degradation, as illustrated by the stability of soluble HLA-G in the presence of US2. Our data shed new light on the modulation of MHC class I expression on cells at the fetal-maternal interface in the context of HCMV infection. More research will be needed to evaluate the immunological consequences of these virus-induced changes in MHC expression.

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CHAPTER 4

Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2-and US11-mediated degradation

Submitted

Subtle sequence variation among MHC class I locus products greatly influences sensitivity to HCMV US2-and US11-mediated degradation.

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Human cytomegalovirus (HCMV) interferes with cellular immune responses by modulating surface expression of MHC class I molecules. Here, we focused on HCMV-encoded US2 and US11, which bind newly synthesized MHC class I heavy chains and support their dislocation into the cytosol for subsequent degradation by proteasomes. Not all MHC class I locus products are equally sensitive to this down-modulation. The aim of this study was to identify which domains, and ultimately which residues, are responsible for the resistance or sensitivity of MHC class I molecules to US2- and US11-mediated down-regulation. We show that, besides ER-lumenal regions, the C-terminus of class I molecules represents an important determinant for allele specificity in US11-mediated degradation. HLA-E becomes sensitive to US11-mediated down-regulation when its cytoplasmic tail is extended. Interestingly, this only requires two additional residues, lysine and valine, at its C-terminus. For US2, the MHC class I allele specificity is largely determined by a small region at the junction of the α2/α3 domain of the heavy chain. It is quite remarkable that minor changes, in only 4 residues, can completely revert the sensitivity of naturally US2-resistant HLA-E molecules. With this study we provide better insights into the features underlying the selectivity in MHC class I down-regulation by US2 and US11.

Human cytomegalovirus (HCMV) establishes persistent infections in human populations worldwide and can give rise to serious disease in immunocompromised individuals. HCMV uses various defense mechanisms to elude the host immune system. Elimination of infected cells by CD8+ T cells can be prevented by down-regulation of viral antigen-presenting MHC class I molecules. In the course of HCMV infection, MHC class I surface expression is affected by several HCMV Unique Short (US) region-encoded proteins, which act at different levels of the antigen processing and presentation pathway (1-5). Antigen presentation is prevented by blocking the supply of peptides through TAP inhibition (US6), by retaining newly synthesized MHC class I molecules in the ER compartment (US3) or by dislocating class I heavy chains back into the cytosol for subsequent degradation by proteasomes (US2 and US11). Efficient down-regulation may very well require the concerted action of several of these US proteins.

A complete reduction of MHC class I expression could have serious consequences for the survival of the virus, as cells lacking MHC class I surface molecules are more susceptible to an NK-cell attack (6). Several proteins encoded within the Unique Long (UL) region of the HCMV genome (UL16, UL18, UL40, UL141) appear to protect infected cells against NK-cell lysis. They either block expression of ligands that activate NK cells (UL16, UL141) or allow expression of ligands that can inhibit NK-cell triggering (UL18, UL40) (7,8).

In general, host cells express HLA-A, -B, -C and -E alleles. The (surface) expression levels of the various locus products is differentially regulated (9,10). HLA-A and -B are generally most abundant at the cell surface. Between the various MHC class I locus products, most sequence variation is found in the region encompassing the antigen-binding groove (11). This generates different restrictions for peptide binding as well as for the variety of peptide display for each allele. MHC class I molecules can serve a dual role, as a ligand for both T and NK cell receptors, but their key task may be biased. HLA-A and –B alleles are probably most powerful in the presentation of foreign peptides. This is supported by a high degree of polymorphism in the world population for these alleles, with 325 different HLA-A, and 592 different HLA-B alleles reported to the IMGT/HLA database sofar. Next comes HLA-C (175 alleles) and the lowest polymorphic is HLA-E (5 alleles) (11). Although there are reports showing that HLA-C and HLA-E can present foreign antigens to T cell receptors, they may primarily serve as interaction partners for NK cell receptors (7,12-17).

Taking into account the functions of the different MHC class I locus products, it is important to know which alleles are affected in HCMV infected cells. Data from

several laboratories have shown that there are allelic differences between MHC class I molecules with respect to sensitivity to US2, US3, US6 and US11, but the picture is still far from complete (18-23). In this study we aimed to characterize the precise regions in MHC class I alleles that determine sensitivity or resistance to these US proteins. This knowledge will also help to predict the down-modulatory effect of US2 and US11 for a broader range of MHC class I alleles.

MATERIALS AND METHODS

Cell lines

J26 cells (H-2^k murine Ltk$^-$ cells expressing human 2m) (24) and the Phoenix amphotropic retroviral producer cell line (American Type Culture Collection, Manassas, VA) were cultured in DMEM (Invitrogen, Breda, The Netherlands), supplemented with 10% FCS (Greiner bv, Alphen aan den Rijn, The Netherlands), 100 U/ml penicillin and 100 g/ml streptomycin and G418 (Invitrogen, Cergy-Pontoise, France). J26 cells expressing HLA-A2 (A*0201), HLA-B7 (B*070201), HLA-B27 (B*270502), HLA-Cw3 (Cw*0304, gift from B. van den Eynde, Brussels, Belgium), HLA-G (G*01011), and HLA-E (E*01033, gift from E. Weiss, Munchen, Germany) were all described previously (19,25).

Antibodies

The following anti-MHC class I mAbs were used for flow cytometry: W6/32 (anti-human MHC I complex) (26), BB7.2, MA2.1 (both anti-HLA-A2) (27), MEM-E/06 (anti-HLA-E; EXBIO Praha, Czech Republic), B1.23.2 (anti-HLA-B and -C) (28), BB7.1 (anti-HLA-B, ATCC), Y-3 (murine MHC class I; ATCC). In most cases, primary mAbs were used in combination with PE-conjugated goat anti-mouse (g m), IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). In some experiments biotinylated-g m Ig was used as second Ab, in combination with streptavidin-conjugated PE as third Ab (PharMingen, Europe). The mAbs MEM-E/02 (denatured HLA-E; EXBIO Praha, Czech Republic), H68.4 (transferrin receptor; Zymed Laboratories, San Francisco, CA) and polyclonal antisera US2-N2 (US2) (19) and US11-N2 (US11) (29) were used for immuno-precipitations. A control experiment was performed with MEM-E/02 to exclude possible cross-reactivity with murine MHC class I. MEM-E/02 only precipitated MHC class I heavy chains in J26 cells transfected with HLA-E and no MHC class I in wild type J26 cells (data not shown).

Construction of plasmids

Plasmid pLUMC9901 (encoding HLA-A*0201 cDNA) (29) was used as template for the construction of HLA-A2delCKV (HLA-A2 with a deletion of residues 340-342). pLUMC9901 and pcDNA-E(sigA2) (encoding cDNA of HLA-E*01033 with signal sequence of HLA-A2; kind gift of E. Weiss, Munchen, Germany) (30) were used as template to construct HLA-A2/E chimeras HLA-A2 $^{1-184}$/E (residues 1-184 of HLA-A2 and rest of HLA-E) and its reverse HLA-E $^{1-184}$/A2, HLA-E(3+c A2) (HLA-E with 3 domain and connecting peptide region of HLA-A2) HLA-E(TM A2) (HLA-E with transmembrane domain of HLA-A2), HLA-E(tail A2) (HLA-E with cytoplasmic tail of HLA-A2) and HLA-A2(tail E) (HLA-A2 with cytoplasmic tail of HLA-E). pcDNA-E (sigA2) was also used to construct the mutants HLA-E ^{QRTD} (HLA-E with residues 180-183, LHLE, replaced by QRTD), HLA-E+KV and HLA-E+ACKV (HLA-E with tail extended with KV or ACKV residues, respectively). pCR-B7 (HLA-B*070201 in vector pCR3.1 (Invitrogen); kind gift of M. Heemskerk, Leiden, The Netherlands) was used as template to construct HLA-B7 ^{ETLQ} (HLA-B7 with residues 177, 178, 180, DK-E, replaced by ET-Q, resulting in the sequence ETLQ at position 177-180). Amplifications were performed using *Isis* polymerase (Q.BIOgene, Illkirch, France). Mutations were introduced using the QuickChange XL Site-directed mutagenesis kit and/or protocol (Stratagene, La Jolla, USA) and chimeric constructs were generated applying the megaprimer method (31). All constructs were fully sequenced to verify the absence of unwanted mutations.

Transfection

J26 cells were transfected with the different MHC class I constructs using Effectene™ Transfection Reagent (Qiagen, Courtaboeuf, France). After 48 hours, stable transfectants were selected by adding 0.4 mg/ml G418 (Invitrogen). Cells were sorted by flow cytometry for expression of the introduced cDNA using MEM-E/06, W6/32, B1.23.2 or BB7.1 mAbs.

Production of retrovirus and transduction

US2 and US11 cDNA fragments were subcloned into the pLZRS-*IRES*-EGFP vector (19,32,33) and used for transfection of amphotropic Phoenix packaging cells to produce retrovirus, as described (19). Cells were transduced with retrovirus using Retronectin (Takara Shuzo, Otsu, Japan)-coated dishes.

Flow cytometry

Cell surface expression of MHC class I molecules as well as EGFP expression in cells transduced with retrovirus were analyzed using flow cytometry as described (19). In cells expressing low levels of human MHC class I (e.g. HLA-E) and/or when indicated, cells were stained in 3 steps to intensify the MHC class I (PE) staining (first with specific anti-MHC class I antibody, then with biotinylated-gαm and finally with streptavidin-conjugated PE). Data are collected from several experiments (generally 3), of which one representative experiment is shown.

Metabolic labeling, immunoprecipitation and SDS-PAGE

Metabolic labeling, immunoprecipitations on denatured samples and SDS-PAGE were performed as described (29). In brief, cells were starved in medium without methionine (Met) or cysteine (Cys) for ~1 hour, labeled with promix (^{35}S Met and Cys), and chased in medium with excess amounts of cold Met and Cys. Where indicated, media were supplemented with proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucinal (ZL_3H). Cells were lysed in a small volume of Nonidet-P40 lysis buffer, containing protease inhibitors. Cell debris was removed by centrifugation and the supernatant was transferred to a new tube to which 1/10 volume of 10% SDS and 1/10 volume of 0.1 M DTT was added. Samples were boiled for 5 min to further denature the proteins. Next, the volume was increased 10 times with non-denaturing buffer, supplemented with protease inhibitors and with 10 mM iodoacetic acid. Immunoprecipitations were performed ≥ 2 h on precleared samples, with Abs pre-coupled to protein A sepharose beads. Samples were separated by SDS-PAGE and displayed via phosphor imaging.

RESULTS

Selectivity of US2 and US11 for MHC class I locus products

US2 and US11 can target specific MHC class I locus products for degradation while preserving surface expression of others (19,21,23,34). In this study we aim to find more information on MHC class I allele specificity of US2 and US11.

Previously, we have found that HLA-A2, HLA-B27, and HLA-G were down-modulated by US2, while surface expression of HLA-B7, HLA-Cw3 and HLA-E was unaffected (32). For US11 it has been shown that it can down-modulate HLA-A2 and HLA-C molecules, but not HLA-G or HLA-E (19,21,23). Although HLA-B locus products are generally believed to be down-regulated by US11, as suggested by a destabilizing effect of US11 on general pools of MHC class I, formal proof is still lacking. To evaluate the stability of individual HLA-B locus products, we used murine J26 cells expressing one particular human MHC class I heavy chain construct and transduced these cells with a retroviral vector encoding both US11 and EGFP. The EGFP expression is used as a marker for transduction (i.e. US11 positive cells). These J26 cells co-express human β2m to allow proper MHC class I complex formation. Figure 1A shows the effect of US11 (EGFP+ cells) on surface expression of HLA-B7, and -B27 compared to the nontransduced (EGFP –) cell population, as analyzed by flow cytometry. It also includes data on HLA-C, -E, and murine alleles to provide a more complete overview in this experimental system. In the US11-expressing cells, surface expression of HLA-B7, HLA-B27, HLA-Cw3 and endogenous murine MHC class I molecules were reduced compared to the non-transduced cells, while HLA-E expression remained unaffected. Control wt-EGFP-expressing retrovirus had no effect on MHC class I cell surface expression (data not shown). Figure 1B shows an overview of the sensitivity to US2 and US11 for all the different MHC class I alleles that have been tested in this experimental system. Representative dot-plot data not shown here, have been presented in our previous studies (refs. 18, 31). This overview shows that there are clear specificity differences between US2 and US11. US2 affects HLA-A2, -B27, and –G and not HLA-B7, -Cw3, -E, or endogenous H-2k, while US11 affects all of these alleles except HLA-E and –G. HLA-E is the only allele that is not affected by either one of these two US proteins.

Residues around the junction of the α2/α3 domains of MHC class I alleles are critical for US2-mediated down-regulation.

The summary in Figure 1B clearly shows that individual human locus products differ in their sensitivity to US2-mediated down-regulation. Crystal structure data of a soluble HLA-A2/US2/β2m complex have shown that at least a region in the α2/α3 domain of HLA-A2 is involved in the interaction with US2 (Figure 2A) (35). These data cannot exclude the possibility that additional interactions with trans-membrane or cytoplasmic tail regions contribute to sensitivity differences between alleles. So, before exploring the effect of sequence variation in this ER-

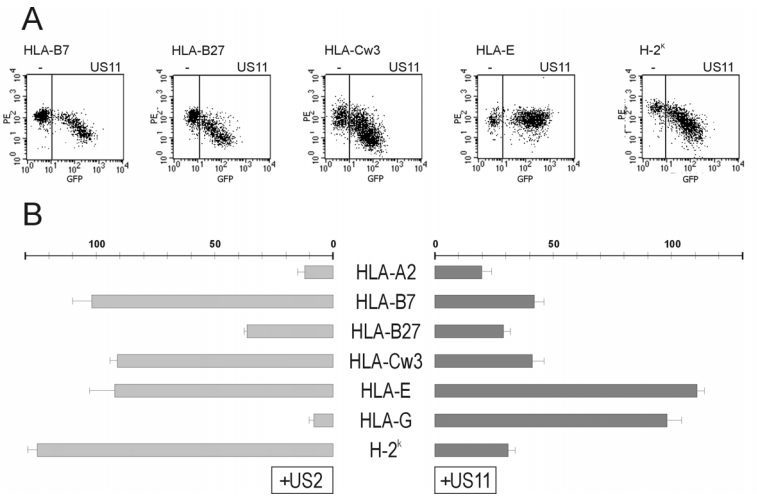

FIGURE 1. Selective down-regulation of HLA class I molecules by US2 and US11. (**A**) Murine J26 cells, transfected with different plasmids encoding HLA class I and transduced with US11-*IRES*-EGFP encoding retrovirus, were analyzed using flow cytometry. The following mAbs were used to stain the different human MHC class I molecules with PE in a 2- or 3-step staining protocol (Y-axis): HLA-B7, -B27, and -Cw3 (W6/32), HLA-E (MEM-E/06, 3-steps) or endogenous H-2^k (Y-3). US11 positive cells are marked by EGFP expression (X-axis). (**B**) Overview of sensitivity of different MHC class I locus products to US2- and US11-mediated down-regulation, as evaluated in the same experimental system in this and in our previous studies (19,32). The effect of US11 on surface staining of MHC class I was calculated by comparing mean PE fluorescence of EGFP negative (defined as 100%) and EGFP positive cells. The averages of calculations of at least two independent experiments are shown, with error bars.

lumenal region, we first addressed this question using chimeras consisting of US2-insensitive HLA-E and US2-sensitive HLA-A2 alleles. We tested the sensitivity of a chimeric molecule in which the ER-lumenal part comprising residues 1-184, is derived from HLA-A2, and the remainder from HLA-E (HLA-A2$^{1-184}$/E). This construct contains all the HLA-A2 residues that are implicated in US2-binding according to the crystal structure data. We also included a reciprocal version of this construct (HLA-E$^{1-184}$/A2). Figure 2B shows that HLA-A2$^{1-184}$/E was almost equally sensitive to US2 as wild type HLA-A2. Likewise, HLA-E$^{1-184}$/A2 was as insensitive as wild type HLA-E. This shows that the sensitivity differences of HLA-A2 and HLA-E are determined by something located between amino acids 1-184 of the ER-lumenal region.

A concordance can be found between US2-insensitive class I alleles (HLA-B7, HLA-E) and variation in the region that for HLA-A2 was shown to be involved in binding to US2 (E177, T178, Q180, R181, T182, D183). To test if sequence variation in this region can indeed account for the observed allelic sensitivity differences, mutants of US2-insensitive HLA-B7 and HLA-E were constructed that resemble their US2-sensitive allelic counterparts (see Figure 2A). Residues at positions 177, 178 and 180 of HLA-B7 were replaced with the corresponding residues E, T and Q of US2-sensitive HLA-B27. Figure 2B shows that this HLA-B7 $^{ET(L)Q}$ mutant had a reduced surface expression in the presence of US2 similar to that observed for HLA-B27, while wild type HLA-B7 remained unaffected. Likewise, a clear sensitivity conversion was observed for an HLA-E mutant with residues 180-183 replaced by QRTD. Whereas wild type HLA-E surface expression was unaffected in US2 expressing cells, surface expression of the mutant HLA-E was clearly reduced. The sensitivity shift was less dramatic, but still clearly visible, when only one mutation, H181R, was introduced into HLA-E.

These flow cytometry data mainly provide information on alterations in surface expression levels in the presence of US2. To exclude the possibility of retention, rather than degradation, being the underlying mechanism for a reduced surface expression, we also evaluated the effect of US2 on the stability of the HLA-E ^{QRTD} mutant by pulse chase analysis (Figure 2C). Like US11, US2 can mediate the retro-translocation of newly synthesized class I heavy chains to the cytosol where they are first deprived of their N-linked glycan through the action of an N-glycanase and subsequently degraded by the proteasome (4,5). This is a very rapid process, taking place from the start of pulse labeling. Figure 2C (left

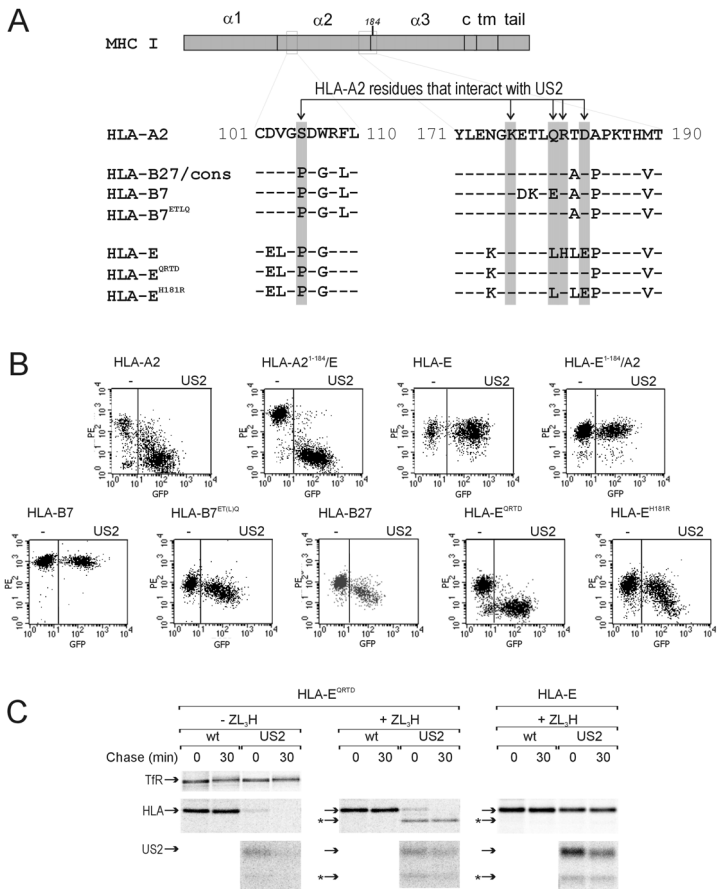

FIGURE 2. Alterations in the α2/α3 domain of MHC class I alleles affect sensitivity to US2-mediated downregu-lation. (**A)** Depicted is HLA-A2 showing ER-lumenal regions (α1- α3 and connec-ting peptide (c)), transmembrane domain (TM) and cytoplasmic tail, as well as an enlargment of the boxed regions (aa 101-110, 171-190) of HLA-A2 with residues directly involved in interaction with US2 marked in grey (according to crystal structure data from Gewurz *et al.*((35)). The US2 binding site of HLA-A2 has been aligned with corresponding regions of other alleles, and their locus (sub)group consensus sequences (cons) obtained from the IMGT/HLA Sequence Database (11). (**B**). J26 cells were transfected with different HLA class I mutants, transduced with US2-*IRES*-EGFP encoding retrovirus and analyzed using flow cytometry. The following mAbs were used to stain the different human MHC class I molecules with PE, in a 3-step staining protocol (Y-axis): HLA-A2, -A2$^{1-184}$/E (BB7.2), HLA-B, HLA-E$^{1-184}$/A2 (W6/32), HLA-E (MEM-E/06). US2 positive cells are marked by EGFP expression (X-axis).(**C**) J26 cells expressing wt HLA-E or HLA-E ^{QRTD} or cells co-expressing US2 were meta-bolically labeled for 10 minutes and chased for 0 or 30 minutes in the absence or presence of proteasome inhibitor ZL_3H. Immuno-precipitations were performed on denatured cell lysates using the following antisera: H68.4 (transferrin receptor [TfR]), MEM/02 (HLA-E) or US2-N2 (US2) Abs. Deglycosylated degradation inter-mediates are marked by an asterisk.

panel) shows that, in the absence of proteasome inhibitors, US2 has a strong destabilizing effect on HLA-E ^{QRTD}; only a small amount of HLA-E ^{QRTD} heavy chains could be immunoprecipitated in US2+ cells at the beginning of the chase and no heavy chains can be recovered after a 30 minute chase, while HLA-E ^{QRTD} remained stable in the absence of US2. Equal amounts of transferrin receptor could be recovered over the chase course in US2+/- samples. In the presence of proteasome inhibitor (+ZL_3H, middle and right panel), deglycosylated degradation intermediates could be recovered for HLA-E ^{QRTD}, which are absent in US2 negative cells and in the US2 positive cells expressing wild type HLA-E. It has to be noted that, in the presence of proteasome inhibitor, dislocated heavy chains remain targets for other cytosolic proteases.

Altogether, these results show that subtle changes in a small region around the junction of the α2/α3

domains of MHC class I can greatly affect their sensitivity to US2-mediated degradation.

In contrast to US2, US11 can discriminate between MHC class I locus products on the basis of their cytoplasmic tail sequences.

For US11 it is still rather unclear which regions of MHC class I molecules determine allelic sensitivity differences to US11-mediated down-regulation. Chimeric HLA-A2/G molecules, consisting of US11-sensitive HLA-A2 and US11-resistant HLA-G alleles, showed that the length of the cytoplasmic tail was the most important determinant for the insensitivity of HLA-G (19). An extension of the short (6 amino acids) HLA-G tail with residues matching the relatively long (33 amino acids) tail of HLA-A2 made it very sensitive to US11-mediated degradation. However, this information cannot directly explain the insensitivity to US11-mediated down-regulation for HLA-E, which has a relatively long cytoplasmic tail of 29 residues. Chimeras of HLA-E and HLA-A2 were constructed to test which regions determine resistance or sensitivity to US11 (see Figure 2A). Like for US2, we first evaluated the effect of US11 on surface expression of the HLA-A2$^{1\text{-}184}$/E and HLA-E$^{1\text{-}184}$/A2 chimeras. Figure 3B shows that the HLA-A2/E chimera consisting of residues 1-184 of HLA-A2 was somewhat down-regulated in US11 positive cells. This chimera was more sensitive to US11 compared to wild type HLA-E, but less sensitive than wild type HLA-A2. HLA-E$^{1\text{-}184}$/A2 on the other hand, was as sensitive to US11 as wild type HLA-A2, with an almost complete reduction

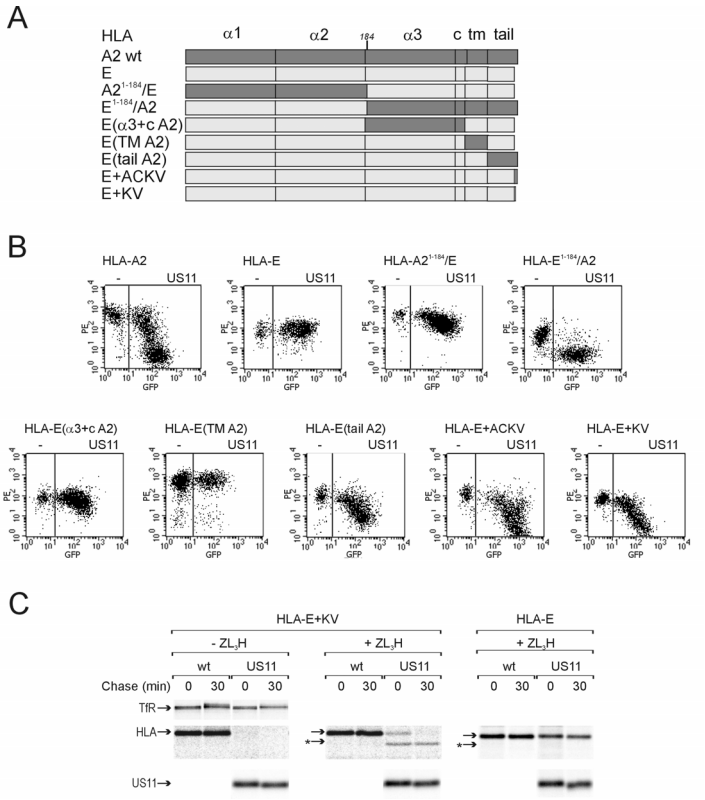

FIGURE 3. Exchange of ER-lumenal domains or extension of the cytoplasmic tail of HLA-E by two residues alter their sensitivity to US11-mediated degradation. (**A**) Overview of HLA-A2/E chimeras showing regions that are exchanged. (**B**) J26 cells transfected with the HLA class I chimeras shown in (A) and transduced with US11-*IRES*-EGFP encoding retrovirus were analyzed using flow cytometry. HLA class I molecules were stained with PE in a 3-step staining protocol (Y-axis) using MEM-E/06 mAb, except for HLA-A2, -A2$^{1\text{-}184}$/E (BB7.2), and HLA-E$^{1\text{-}184}$/A2, -E-α3+c A2 which were stained with W6/32 mAb. US11 positive cells are marked by EGFP expression (X-axis). (**C**) J26 cells expressing wt HLA-E or HLA-E+KV or cells co-expressing US11 were metabolically labeled for 10 minutes and chased for 0 or 30 minutes in the absence or presence of proteasome inhibitor ZL_3H. Immuno-precipitations were performed on denatured cell lysates using the following antisera: H68.4 (transferrin receptor [TfR]), MEM/02 (HLA-E) or US11-N2 (US11) mAbs.

of surface expression. We then tested what region of HLA-A2 was responsible for this down-regulation of HLA-E$^{1-184}$/A2. Replacement of the α3 domain and connecting peptide region with the corresponding domains of HLA-A2 (HLA-E(α3+c A2)) caused a slight reduction in surface expression, observed in the highest EGFP (and US11)-expressing population only. Replacement of the transmembrane region (HLA-E(TM A2)) showed no effect. Exchanging the cytoplasmic tail (HLA-E(tail A2)) however, resulted in a remarkable increase in sensitivity to US11. This indicated once again that the cytoplasmic tail can be an important determinant for US11-mediated down-regulation.

A more detailed analysis was performed to determine which element of the HLA-A2 tail made the HLA-E(tail A2) chimera sensitive to US11. In a previous report, we have shown that MHC class I heavy chains are less sensitive to US11-mediated down-regulation when the cytosolic tail lacks the lysine and valine residues at the extreme end (19). HLA-E molecules have a slightly shorter tail than HLA-A2 as they lack C-terminal –ACKV residues. We tested whether addition of these residues could increase the sensitivity of HLA-E to US11-mediated down-regulation. Figure 3B shows that an extension of the cytoplasmic tail with–ACKV or –KV residues markedly reduced surface expression of these HLA-E mutants in US11 expressing cells.

We also evaluated the effect of US11 on the stability of HLA-E+KV in pulse chase experiments (Figure 3C). In the absence of proteasome inhibitor (left panel), HLA-E+KV heavy chains remained stable over time in US11 negative cells, but were clearly unstable in the presence of US11. This effect of US11 is specific for class I HC's, as the amount of transferrin receptor in these cells was not reduced. In the presence of proteasome inhibitor (middle and right panel), a deglycosylated breakdown intermediate was observed only in cells expressing US11 and only for HLA-E with the –KV extension, but not for wild type HLA-E.

From these results we can conclude that HLA-E, like HLA-G, becomes sensitive to US11-mediated degradation when its tail is extended with HLA-A2 tail residues. This indicates that the cytoplasmic tail can be an important determinant for sensitivity to US11. Interestingly, it only required two extra residues at its C-terminus for HLA-E to become completely sensitive to US11. Clearly, the tail is not the only determinant, as the α1/α2 region and to a lesser extent the α3/connecting peptide region also contribute to the efficiency of US11-mediated down-regulation of MHC class I molecules.

C-terminal lysine and valine residues influence efficiency, but are not essential for US11-mediated down-regulation of MHC class I molecules.

When looking at the cytoplasmic tail sequences of MHC class I locus products, it becomes evident that other class I molecules besides HLA-G and HLA-E (e.g. HLA-B molecules), lack lysine and valine

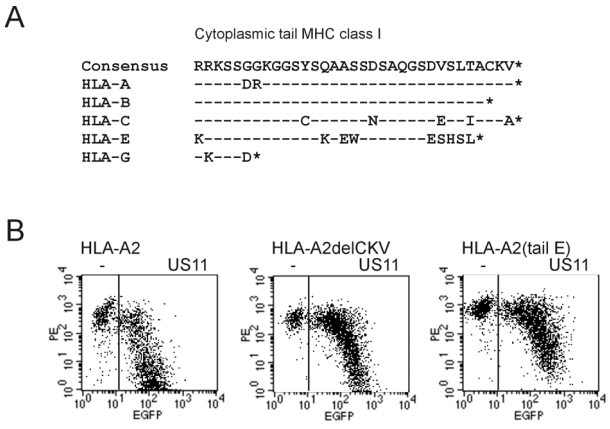

FIGURE 4. Cytoplasmic tail residues of MHC class I molecules determine efficiency of US11-mediated down-regulation. (**A**) Alignment of amino acid sequences of the cytoplasmic tail region of different MHC class I locus products consensus sequences, obtained from the IMGT/HLA Sequence Database (11). (**B**) J26 cells expressing wt HLA-A2, HLA-A2delCKV, or HLA-A2(tailE) were transduced with US11-*IRES*-EGFP encoding retrovirus and analyzed using flow cytometry. HLA class I molecules were stained with PE in a three step staining protocol using BB7.2 as first mAb (Y-axis). US11 positive cells are marked by EGFP expression (X-axis).

residues at their C-terminal tail (Figure 4A). Figure 3 already showed that ER-lumenal regions influence the efficiency of down-modulation by US11. To evaluate if this region could also be sufficient for down-modulation of HLA-A2 molecules in the absence of lysine and valine residues, we tested two different HLA-A2 mutants. One mutant (HLA-A2delCKV) lacks the last three C-terminal residues, as do HLA-B alleles, and the other mutant has the same tail as HLA-E molecules (HLA-A2(tailE)). Figure 4B shows that other regions than the cytoplasmic tail KV residues can mediate sufficient interactions for a US11-mediated reduction in surface expression, as HLA-A2 mutants without these residues were also down-regulated. Interestingly, the efficiency of down-regulation of class I molecules lacking (A)CKV residues seems to be somewhat lower than of those with these residues at the extreme end of the tail. In general, the level of down-modulation is lower in the low EGFP positive cells and increases with rising EGFP (i.e. US11) levels. In cells expressing highly sensitive wild type HLA-A2, effective reduction in surface expression can also be observed in cells expressing relatively low levels of EGFP.

These results show that the C-terminal KV residues do not necessarily function as strong determinants for sensitivity to US11 for all locus products. As opposed to the crucial role of KV residues in US11-mediated down-regulation of HLA-E molecules, they are less important for down-regulation of other MHC class I locus products. This is shown for HLA-A2 molecules, where the (C)KV tail residues could be removed without completely loosing sensitivity to US11. It can also explain the observed down-regulation of HLA-B molecules (Figure 1A), which naturally lack CKV residues. However, the presence or absence of these KV residues can nevertheless influence the efficiency of down-modulation by US11, and can determine the levels of US11 that are required for sufficient modulation of antigen presentation by MHC class I.

DISCUSSION

HCMV encodes several proteins that interfere with cross talk between infected host cells and host immune effector cells through modulation of surface expression of MHC class I molecules. The success of immune escape by HCMV through modulation of MHC class I surface expression is likely to be influenced by the efficiency, as well as by the specificity of this down-modulation by the different US proteins. US3 mainly affects surface expression of tapasin-dependent MHC class I alleles (20). By blocking TAP, US6 prevents peptide transport into the ER and subsequent peptide loading. This affects surface expression of all MHC class I alleles (2,3). In spite of this, surface expression of HLA-E molecules is preserved by supplying it with a TAP-independent peptide source (30,36). In this study we focused on US2 and US11, which both target different sets of newly synthesized MHC class I molecules for degradation. In this study we further clarify how, and to what extend, US2 and US11 can contribute to the efficiency and specificity of MHC class I down-regulation.

We and others have found that US2 differentially affects surface levels of individual human MHC class I locus products (23,32,34). Based on crystal structure data of HLA-A2/β2m/US2 and sequence alignments for the region of class I implicated in US2 binding, we hypothesized that allelic variation in the $\alpha 2/\alpha 3$ ER-lumenal region could form an explanation for the resistance of HLA-B7, HLA-Cw3 and HLA-E (35). In the present study, we tested this hypothesis to see if this would result in a more reliable prediction of US2 sensitivity of, as of yet, untested MHC class I alleles.

Using chimeras derived from US2-sensitive (HLA-A2) and –insensitive (HLA-E) alleles, we found that there are no other regions in HLA molecules, outside the ER-lumenal region implicated in US2-binding, that contribute to US2-mediated down-regulation. Sequence alignments of HLA-B27 and HLA-B7 also point to a role of the ER-lumenal region in selective down-regulation of only HLA-B27 and not HLA-B7, as there are no differences in the amino acid sequence outside the ER-lumenal region between these two alleles.

We then investigated if we could convert the resistance of HLA-B7 and HLA-E by replacing those residues that are assumed to prohibit an interaction with US2, with the corresponding residues found in US2-sensitive alleles (as described in figure 2A). The HLA-B7 $^{ET(L)Q}$ and HLA-E ^{QRTD} mutants showed that residues in this region are indeed important sensitivity determinants, as this alteration of only 3 or 4 residues clearly affected the surface expression of these mutants in the presence of US2. We showed for HLA-E ^{QRTD} that it is targeted for degradation, thereby excluding the possibility that retention is the underlying mechanism for the observed down-modulation.

We also tried to find an explanation for the resistance of HLA-Cw3 alleles to US2-mediated down-regulation. The presence of particular residues at positions 183 (E) and 184 (D) appeared not to be responsible for its resistance. This is supported by recent data showing that HLA-C molecules (HLA-Cw7 and –Cw2), which all have E183 and D184, can nevertheless be down-regulated by US2 ((34); our own unpublished results). Nonetheless, sequence variation at this site may still affect the efficiency of down-modulation, as US2-resistant HLA-Cw3 alleles became somewhat more sensitive when E183D, H184P mutations were introduced (unpublished results). However, the presence of a positively charged lysine residue at position 173 is the most likely explanation for the resistance to US2-mediated down-regulation of HLA-Cw3, since US2-sensitive alleles, including the majority of HLA-C alleles, have a negatively charged glutamic acid at position 173.

For US11, the only MHC class I locus products completely insensitive to down-modulation were HLA-G and HLA-E (Figure 1). Interestingly, all that is required to confer sensitivity to these two MHC class I locus products, is an extension of their cytoplasmic tail. Previous studies have shown that the length of the class I cytoplasmic tail is very important. Tailless HLA-A2 molecules (with a tail shortened to either 4 or 6 amino acids) could no longer be targeted for degradation by US11 (19,37). Conversely, HLA-G molecules naturally have a tail of 6 residues and an extension of this tail with 27 HLA-A2 tail residues resulted in a very efficient degradation of these mutants in US11 positive cells (19). Interestingly, an extension of the tail of HLA-G with 25 HLA-A2 tail residues did not give this result. Apparently, the C-terminal lysine and valine residues were essential for degradation. HLA-E has a cytoplasmic tail that lacks only 4 residues compared to HLA-A molecules (see Figure 4A). Interestingly, HLA-E required only 2 extra residues (lysine and valine) to become sensitive to US11. This indicates that the length of the tail can be an important determinant for sensitivity differences among MHC class I locus products. HLA-B molecules, on the other hand, also lack the lysine and valine residues at their C-terminus, but are nevertheless down-modulated by US11. We showed in figure 4 that the lysine and valine residues are not essential for down-regulation of all haplotypes, as HLA-A2 with a tail as long as that of HLA-B molecules (HLA-A2delCKV) or with the HLA-E tail could still be down-modulated by US11. These residues can, however, determine the effectiveness or threshold for down-regulation, as HLA-A2delCKV and HLA-A2(tail E) seemed to require higher levels of US11 than wild type HLA-A2 for a similar down-regulatory effect. At the same time, these data show that ER-lumenal residues also influence sensitivity to US11. This is further supported by our findings that the exchange of $\alpha1/\alpha2$ domains or of the $\alpha3$ domain of HLA-E with those of HLA-A2 could, to some extent, also change the efficiency of down-modulation by US11.

Whereas specificity of US2-mediated down-modulation seems to rely mostly on a region at the junction of the $\alpha2/\alpha3$ domain, these data indicate that the conditions are different and more complicated for US11. Although ER-lumenal residues do play a role, replacement of residues LHLE in HLA-E by QRTD did not affect its sensitivity to US11 (unpublished results). Also, US2 does not require MHC class I tail residues, but US11-mediated down-modulation depends largely on this region. In principal, all MHC class I cytoplasmic domains, with the exception of HLA-G, bear the essential residues necessary for US11 to target them for degradation. Our data indicate that a minimum of 29 class I tail residues has to go with either a favorable ER-lumenal region, or with lysine and valine tail residues in order to see sufficient down-modulation. A favorable ER-lumenal region may bypass the function of the KV residues through a prolonged and/or stronger interaction with US11, thereby increasing the chances of dislocation and subsequent degradation.

The function of the KV, as well as of the other tail residues is still unclear. Enhancement of down-regulation by the KV residues may rely on the lysine, functioning as a potential ubiquitination site. It is known that ubiquitination is essential for US11-mediated targeting for dislocation and subsequent degradation of MHC class I molecules (29,38). Although a study by Shamu et al. showed that lysine residues in the tail of HLA-A2 are not essential for US11-mediated degradation, elimination of HLA-A2 without lysine residues in the tail seemed to be retarded compared to wild type HLA-A2 (39). Alternatively, the KV residues may merely facilitate access of components of the dislocation/degradation machinery to essential residues residing within another region of the tail. Phosphorylation can be an important signal for docking of E3 ligases, which in turn can ubiquitinate their substrates (40). The tail of MHC class I molecules encodes several potential phosphorylations sites, one of which, S335, is a known phosphorylation site that can be found in all

class I locus products (41,42). We have mutated this residue S335 in HLA-A2, as well as two other potential phosphorylation sites close by (S328, S332) and replaced them with alanine. However, these mutants did not behave any differently from wild type HLA-A2, in the presence of US11 (unpublished results). More research will be required to unravel why the tail is essential for US11 to mark MHC class I molecules as substrate for the ubiquitination machinery.

All in all, we showed here that sequence variation around the region comprising residues 176-183 accounts for sensitivity differences of MHC class I locus products to US2-mediated degradation. This knowledge provides a valuable tool to predict the effect of US2 on a broader range of HLA class I molecules. For US11, we showed that not only ER-lumenal regions, but also cytosolic tail residues are important determinants for the outcome of the down-modulatory effect of US11 on different class I locus products. The length of the tail can explain the insensitivity of HLA-G and HLA-E, and may also determine the efficiency by which other HLA class I molecules are down-modulated. More research is still required to define which regions in the α1, α2 and α3 domains of MHC class I molecules are also playing a role.

It is remarkable that the difference between complete resistance and full sensitivity of HLA-E alleles to US2 and US11-mediated degradation relies on as few as 2-4 residues. It is known that a preserved surface expression of HLA-E supports immune escape from NK cell attack of HCMV infected cells (34,36,43). From this point of view, it would be beneficial for the host to reduce its HLA-E surface levels in HCMV infected cells. As mentioned before, it requires only small modifications within HLA-E to render this molecule sensitive to US2 or US11. Despite a long co-evolution of virus and host, HLA-E demonstrates limited polymorphism. This may imply that the residues determining resistance to these viral proteins are essential for interactions of HLA-E with components of the antigen presentation pathway and/or its biological function.

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CHAPTER 5

Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol

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Ubiquitination is essential for human cytomegalovirus US11 mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol

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Human cytomegalovirus encodes two glycoproteins, US2 and US11, which cause rapid degradation of MHC class I molecules, thus preventing recognition of virus-infected cells by the immune system. This degradation process involves retrograde transport or 'dislocation' of MHC class I molecules from the endoplasmic reticulum (ER) to the cytosol, where they are deglycosylated by an N-glycanase and degraded by the proteasome. At present it is unknown whether ubiquitination is required for US2- and US11-mediated dislocation and degradation of MHC class I molecules. Here, we show that in E36ts20 hamster cells, which contain a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, US11-mediated degradation of MHC class I molecules is strongly impaired at the non-permissive temperature, indicating the necessity for ubiquitination in this process. We next addressed the question of whether ubiquitination is a condition for the retrograde movement of MHC class I molecules from the ER to the cytosol, or whether ubiquitination is merely required for recognition of dislocated MHC class I molecules by the proteasome. In the absence of a functional ubiquitin system, complexes of US11 and MHC class I molecules accumulate in the ER. In this state the membrane topology of MHC class I molecules does not significantly change, as judged from proteinase K digestions. Thus the results indicate that a functional ubiquitin system is essential for dislocation of MHC class I molecules from the ER to the cytosol.

The proteasome mediates degradation not only of cytosolic and nuclear proteins [1,2], but also of proteins that reside in the ER [3,4]. This discovery implied that ER proteins destined for degradation must be transported, or "dislocated", back to the cytosol for them to access the degradation machinery. Dislocation and subsequent degradation by the proteasome has been observed for an increasing number of ER proteins in several different organisms (reviewed in [2-5]). Of particular interest is the degradation of MHC class I molecules, which are neither misfolded, nor destined for degradation by cellular signals like most known substrates, but which are targeted for degradation by either of two human cytomegalovirus (HCMV)-encoded glycoproteins, US2 or US11 [6,7].

Proteasomes were implicated in this degradation process based on the use of proteasome inhibitors, which caused accumulation of deglycosylated MHC class I breakdown intermediates in the cytosol [6,7]. Such cytosolic deglycosylated intermediates were also found in the course of degradation of T-cell receptor (TCR)α-chains transiently expressed in non-T cells, using proteasome inhibitors [8,9]. Based on co-precipitation of deglycosylated MHC class I heavy chain intermediates with sec61ß [7], the translocon is probably involved in both anterograde and retrograde transport of proteins across the ER membrane. Experiments involving sec61p mutants in yeast support this notion [10-12].

Covalent attachment of ubiquitin chains to lysine residues is the main mode of targeting proteins to proteasomes. Ubiquitinated proteins are recognized by subunits of the 19S cap of the 26S proteasome [2,4]. Involvement of ubiquitination in degradation of ER proteins by the proteasome would therefore be anticipated, and indeed ubiquitination was shown to be essential for degradation of several ER substrates [13-18]. Some substrates, however, are degraded by the proteasome in a ubiquitin-independent manner [19-21].

At present, it is unknown whether ubiquitination is required for US2- and US11-mediated dislocation and degradation of MHC class I molecules. Shamu and co-workers showed that a proportion of the MHC class I heavy chains are ubiquitinated in US11-expressing cells, but their results failed to show whether

ubiquitination is a condition for dislocation or whether it is merely a consequence of dislocation to the cytosol [22].

The attachment of multiple ubiquitin molecules to proteins involves the action of three enzymes, the ubiquitin-activating enzyme, designated E1, a ubiquitin-conjugating/carrier enzyme or E2, and a ubiquitin ligase or E3 [2]. We used the E36-ts20 hamster cell line, which contains a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, to monitor the effects of a disrupted ubiquitin system on US11-mediated degradation of MHC class I molecules. The results indicate that the early step of dislocation of MHC class I molecules from the ER to the cytosol is blocked when the ubiquitin system is not functional. This suggests dual ubiquitin dependence, since it is anticipated that ubiquitination is also needed for recognition of the degradation substrate by the proteasome at a later stage of the degradation pathway.

MATERIALS AND METHODS

Materials

The hamster cell lines E36 and E36ts20 (the latter referred to as ts20 throughout the rest of the article) [23] were maintained at 33°C under an air/ CO_2 (19:1) atmosphere in minimal essential medium (MEMα; Gibco BRL) supplemented with 10% (v/v) foetal calf serum (Greiner), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Gibco BRL).

A polyclonal antiserum against the cytoplasmic tail of HLA-A2 was produced in rabbits using the synthetic peptide KGGSYSQAASSDSAQGSD. Polyclonal antiserum against US11 was raised in rabbits using the synthetic peptide LSLTLFDEPPPLVETEPL, derived from the cytoplasmic tail of US11. Polyclonal rabbit serum specific for unfolded MHC class I heavy chains has been described [24], as well as W6/32 monoclonal antiserum, specific for assembled MHC class I heavy chain-β_2m complexes [25], and monoclonal antiserum HCA2 against the lumenal domain of HLA-A2 heavy chains [26].

The proteasome inhibitors carboxybenzyl-leucyl-leucyl-leucinal (ZL_3H) and carboxyl benzyl-leucyl-leucyl-leucyl vinylsulfone (ZLVS) were from Peptide Institute, Inc. (Japan) and used in a final concentration of 20 µM.

A pcDNA3 derivative, encoding HLA-A0201 under control of the CMV promoter, was constructed as follows. The plasmid pSRα1Neo-HLA-A2, provided by Dr J. Alejandro Madrigal (The Anthony Nolan Research Centre, The Royal Free Hospital, Londen, UK), was digested with XhoII, after which the fragment encompassing the HLA-A2 coding region was rendered blunt by digestion of the 5' protruding ends. The blunt-ended fragment was digested with HindIII and ligated with the large EcoRV-HindIII fragment of pSP72 (Promega). The HLA coding region was then re-isolated as a BglII-XhoI fragment and cloned into BamHI-XhoI digested pcDNA3 (Invitrogen). The resulting plasmid was designated pLUMC9901.

The plasmid containing US11 and the puromycin resistance gene (pIE-puro US11) has been described [27].

Stable transfection of HLA-A2 and US11 into E36 and ts20 cell lines

The plasmids containing HLA-A2 and US11 were transfected separately or together into ts20 and E36 cells, which were both kept at 30-33ºC, using Fugene (Roche) according to the manufacturer's directions. After 48 hours, G418 (Geneticin; Gibco BRL) or puromycin (ICN) was added to the transfected cells at amounts that killed untransfected cells within a few days. Clones were tested for expression of the genes of interest by radiolabelling and immunoprecipitation (see below).

Pre-incubation of cells at different temperatures

Prior to pulse-chase analysis cells were pre-incubated at 40ºC (the non-permissive temperature) or other temperatures as indicated in the figures. Culture flasks containing cells were transferred from the stove to a closed waterbath at the chosen temperature, in which they were incubated for 2 hrs. Starvation and pulse-chase incubations were all performed at the same temperature.

Pulse-chase analysis, immunoprecipitation and SDS/ PAGE

After trypsinization, suspended cells were starved in RPMI 1640 medium (BioWhittaker) without methionine and cysteine for approx. 1 h at either 33ºC or 40ºC. The proteasome inhibitor ZL_3H was added where

indicated. The cells were metabolically labelled with 250 µCi ^{35}S-labelled Redivue Promix (a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine; Amersham) per 10^7 cells in starvation medium (pulse). For chase samples radioactive medium was replaced with RPMI 1640 medium supplemented with 1mM methionine and 0.1mM cysteine. Pulsed and chased cells were lysed in Nonidet P-40 (NP40) containing lysis buffer as described in [24].

Before immunoprecipitation, two subsequent preclears were performed using normal rabbit and normal mouse sera pre-coupled with mixed (1:1) Protein A-Sepharose and Protein G-Sepharose beads. Immunoprecipitation was performed on the precleared lysate for 2-4 hours at 4ºC using specific antiserum pre-coupled to Protein A/G-Sepharose beads. Beads were washed with NET-buffer [50 mM Tris/HCl, pH 7.4/ 150 mM NaCl/ 5 mM EDTA/ 0.5% (v/v) NP-40] supplemented with 0.1 % SDS and subsequently boiled in sample buffer [40 mM Tris/HCl, pH8.0/ 4 mM EDTA/ 8% (w/v) SDS/ 40% (v/v) glycerol/ 0.1% Bromophenol Blue] for 5-10 min. Samples were loaded on SDS/polyacrylamide gels and run overnight. Gels were dried and exposed to a storage PhosphorImaging screen, which was scanned in a Personal Molecular Imager FX and analysed with Quantity One software (BioRad).

FACS analysis of cell surface expression of MHC class I molecules

FACS analysis of cell surface expression of HLA-A2 was performed as described by Ressing et al. [28] using W6/32 antiserum and goat-anti-mouse-FITC conjugate (Jackson, WA, USA).

Infection with vaccinia virus

Infection with vaccinia virus was performed as described previously [29]. Briefly, E36 and ts20 were infected simultaneously with recombinant vaccinia viruses expressing HLA-A2 (vvA2) and HCMV US11 (vvUS11), respectively. Cells (10^6/ml in RPMI without serum) were infected with virus at a multiplicity of infection of 1 for vvA2 and 1.5 for vvUS11. After 1 h of infection, complete medium was added to the infected cells. Cells were subsequently incubated at 40ºC for 3 h followed by a 1 h starvation period in media lacking methionine and cysteine in the presence of proteasome inhibitor where indicated. Pulse-chase experiments were performed as described above.

Subcellular fractionation

Fractionation of cells was performed essentially as described by Wiertz et al. [6]. In brief, about 10^7 cells were starved and labelled for 30 minutes with ^{35}S Redivue Promix as described above. Cells were washed and resuspended in 1 ml of homogenization buffer [0.25 M sucrose/ 10 mM triethanolamine/ 10 mM potassium acetate/ 1 mM EDTA, pH 7.6], supplemented with protease inhibitors leupeptin (0.1 mM) and AEBSF (10 mM)). Cells were placed on ice and homogenized in a Dounce homogenizer (50 strokes) with a tight-fitting pestle. The homogenate was spun for 10 minutes at 4ºC and 1000 *g* in an Eppendorf centrifuge. The pellet was saved and the supernatant was spun for 30 minutes at 4ºC and 10,000 *g*. Again the pellet was saved and the supernatant was spun for 1h at 4ºC and 100,000 *g*. The latter two centrifugations were performed in a TLA 120.2 fixed-angle rotor, operated in a Beckman Optima® TLX ultracentrifuge. All pellets were resuspended in NP-40 lysis buffer. HLA-A2 and US11 were immunoprecipitated simultaneously from the solubilized pellets and the 100,000 *g* supernatant by immunoprecipitation and separated by SDS-PAGE. PhosphorImaging was performed as described above.

Proteinase K digestions

After pre-incubation at 30ºC or 40ºC, cells were labelled with ^{35}S-Redivue Promix as described above and subsequently resuspended in 200 µl of cold permeabilization buffer (containing 25 mM HEPES pH 7.2, 115 mM potassium acetate, 5 mM sodium acetate, 2.5 mM $MgCl_2$, and 0.5 mM EGTA). Proteinase K (Life Technologies) was added in concentrations as indicated and digitonin (Calbiochem) was added in concentrations that were always 100 times higher than the proteinase K concentrations used. The cells were incubated with the digestion mix at 4ºC for 20 minutes. Proteolysis was stopped by centrifuging cells at 14000 *g* for 10 minutes and resuspending the pellets in 1 ml NP-40 lysis buffer containing 2mM PMSF. For denaturing immunoprecipitations pellets were resuspended in 150 µl lysis buffer containing 2 mM PMSF and 1% (w/v) SDS and boiled for 5 min., after which 800 µl lysis buffer was added. Immuno-precipitations, SDS/PAGE and Phosphor-Imaging were performed as described above.

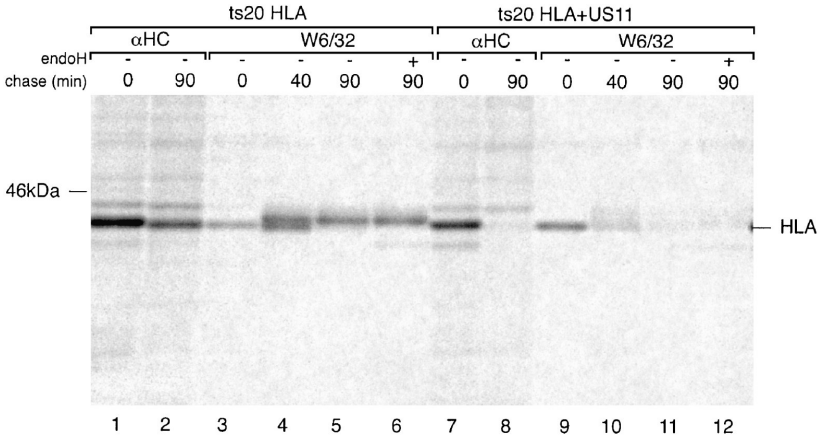

Figure 1. US11 mediated degradation of HLA-A2 in ts20 hamster cells. ts20 cells stably transfected with HLA-A2 alone (ts20 HLA), or both HLA-A2 and US11 (ts20 HLA+US11) were pulse-labelled with [^{35}S]methionine/ [^{35}S]cysteine as described in Materials and Methods for 8 min and chased for the periods indicated at 33°C. Lysates were split and subjected to immunoprecipitation with polyclonal antiserum against the unfolded MHC class I heavy chain (αHC), or conformation dependent antiserum (W6/32), which recognizes the complex of MHC class I heavy chain with β_2m and peptide. Samples in lanes 6 and 12 were treated with endoglycosydase H (endoH). The immunoprecipitates were separated on an SDS/polyacrylamide (10%) gel. The experiment was repeated twice with very similar results, the data shown here are from one of these experiments.

RESULTS

Reconstitution of US11 mediated breakdown of HLA-A2 in hamster cells

The hamster cell line ts20, containing a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, and the parental cell line E36 [23] were stably transfected with human HLA-A2, HCMV US11, or both genes together (see Materials and Methods).

HLA-A2 transfected into ts20 cells is stable at the permissive temperature of 33ºC (Figure 1), and matures from free heavy chains detected by rabbit anti-heavy-chain serum (Figure 1, lanes 1 and 2) to mature, fully assembled molecules as detected by the conformation-dependent antibody W6/32 (Figure 1, lanes 3-5). For HLA-A2, W6/32 reactivity not only requires association with β_2m but also binding of antigenic peptide [25]. W6/32 reactive MHC class I molecules obtained after 90 minutes of chase are endoglycanase H (EndoH)-resistant (Figure 1, lane 6), indicating their transport to the Golgi apparatus. Cell surface expression of transfected HLA-A2 molecules was analysed using flow cytometry (Figure 2). Figure 2 (B) shows that ts20 cells transfected with HLA-A2 express these molecules on their surface. Taken together the data indicate that HLA-A2 is stably expressed in hamster cells, associates with hamster β_2m, acquires antigenic peptides and is transported to the cell surface at 33ºC.

When US11 was co-transfected together with HLA-A2 into ts20 cells, the MHC class I molecules were degraded at the permissive temperature of 33ºC, as assessed by immunoprecipitations with either polyclonal rabbit anti-(MHC class I heavy chain) serum (Figure 1, lanes 7 and 8) or the conformation dependent antibody W6/32 (Figure 1, lanes 9-12). The amount of surface-expressed HLA-A2 was dramatically lower in the cells that express HLA-A2 and US11 (Figure 2C), which is attributed to efficient degradation of HLA-A2 at the permissive temperature.

Very similar results were obtained with E36 cells transfected with HLA-A2 and/or US11 (see below). Pulse-chase analysis of ts20 cells transfected with US11 alone showed that endogenous hamster MHC class I molecules are not degraded (results not shown).

Inhibition of the proteasome interferes with dislocation and degradation of HLA-A2

Treatment of E36 HLA+US11 cells (Figure 3) and ts20 HLA+US11 cells (results not shown) with the proteasome inhibitor ZL_3H delays US11 mediated degradation of MHC class I molecules and causes a

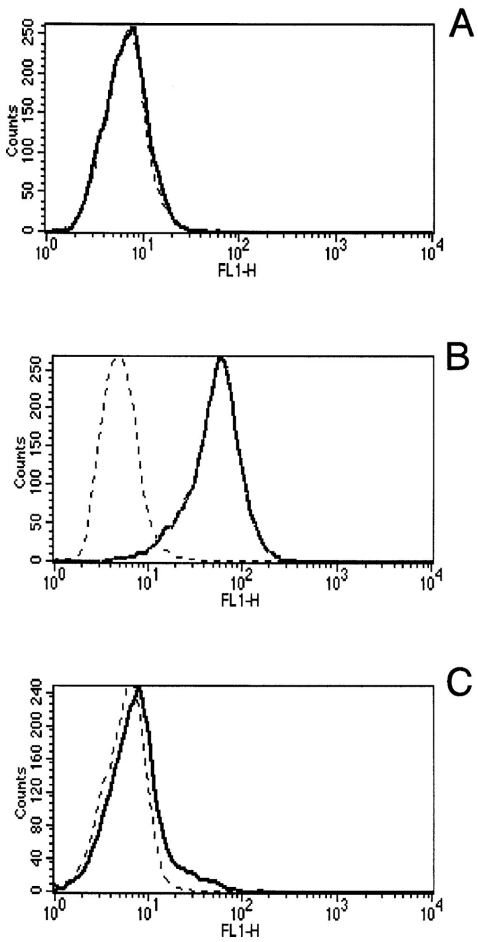

Figure 2. HLA-A2 surface expression is down-regulated in ts20 cells expressing US11. A FACS experiment was performed to assess the cell surface expression of HLA-A2 in different cell lines. At least 10^5 cells were sorted per measurement. The *x* axes represent fluorescence intensity (arbitrary units) and *y* axes represent cell counts. Cells grown at 33°C were incubated with W6/32 antiserum and subsequently with goat-anti-mouse-FITC respectively (black lines). As a control, cells were incubated with goat anti-mouse-FITC alone (dotted lines); (**A**) Wild type ts20 cells; (**B**) ts20 cells transfected with HLA-A2; (**C**) ts20 cells transfected with HLA-A2 and US11.

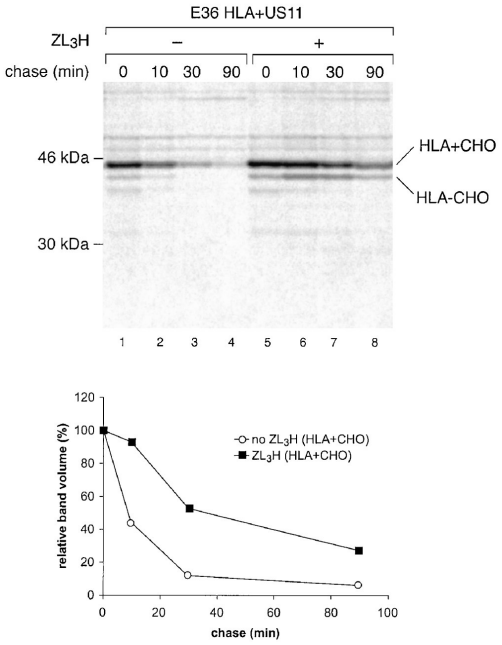

Figure 3. Effect of proteasome inhibitor on US11 mediated degradation of HLA-A2 in E36 cells. E36 HLA+US11 cells were pulse-labelled (10 min) with [^{35}S]methionine/ [^{35}S]cysteine as described in Materials and Methods and chased for 0, 10, 30, and 90 min at 33°C, with or without the proteasome inhibitor ZL_3H. HLA-A2 molecules were immunoprecipitated with antiserum against the cytoplasmic tail of MHC class I molecules and separated on an SDS/polyacrylamide (10%) gel. In the graph, relative volumes of bands representing HLA-A2 molecules with carbohydrate (HLA+CHO) were plotted (measured in arbitrary units, counts/mm^2). The experiment was performed twice with similar results; this figure represents one such experiment.

deglycosylated intermediate to accumulate in the cytosol. In the experiment shown in Figure 3 an antiserum against the cytoplasmic tail of HLA-A2 was used for immunoprecipitation, which recognizes both folded and unfolded molecules.

In the graph on Figure 3 the relative amounts of glycosylated HLA molecules only are represented. The disappearance of glycosylated HLA molecules is a measure of the dislocation of HLA molecules to the cytosol, where they are degraded (in the absence of ZL_3H) or accumulate as deglycosylated breakdown intermediates (in the presence of ZL_3H). The graph indicates that inhibition of the proteolytic activity of the proteasome also inhibits the dislocation process.

At later timepoints in the chase (> 30 minutes) the deglycosylated HLA molecules also begin to disappear (Figure 3, lane 8). It is not very likely that this dis-appearance is related to the concentrations of proteasome inhibitor being too low. Degradation of the deglycosylated breakdown intermediate has also been observed in other cell lines, at higher concentrations of proteasome inhibitor (unpublished observations). Rather, at later time points cytosolic deglycosylated HLA molecules may be degraded by other proteases,

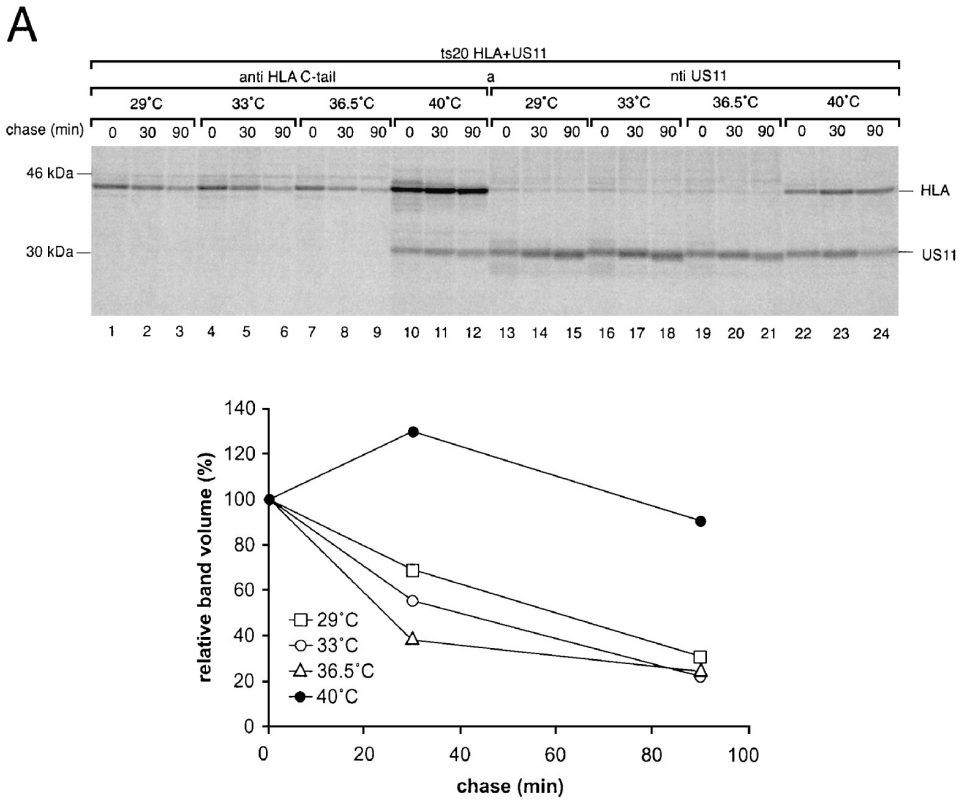

Figure 4. Ubiquitination is required for US11 mediated degradation of MHC class I heavy chains. (**A**) ts20 HLA+US11 cells were pre-incubated at the indicated temperatures for 2 h and subsequently starved, pulse-labelled for 8 min with [^{35}S]methionine/ [^{35}S]cysteine as described in Materials and Methods, and chased for 0, 30 and 90 min at the same temperature, as shown. Immunoprecipitations were performed sequentially with antiserum against the cytoplasmic tail of MHC class I molecules (lanes 1-12) and anti-US11 serum (lanes 13-24), respectively. Samples were separated by SDS/PAGE (10% gel) and a PhosphorImage was generated. The panel shows one experiment that was performed five times in a comparable fashion, all with similar results. (**B**) Pulse-chase analysis of E36 HLA+US11 cells (10 min pulse labelling, chase times as indicated) at 33°C and 40°C using antiserum against the cytoplasmic tail of MHC class I molecules (lanes 1-8) and anti-US11 serum respectively (lanes 9-12). Samples were separated by SDS/PAGE (10% gel) and a PhosphorImage was generated. The experiment was performed twice, with similar results; this figure represents one such experiment. For (**A**) and (**B**), in the graphs relative volumes of HLA-A2 bands, from lanes (**A**) 1-12 and (**B**) 1-8, were plotted (measured in arbitrary units, counts/mm^2) (**C**) E36 and ts20 cells were infected with vaccinia viruses expressing HLA-A2 (lanes 1 and 2), or simultaneously with vaccinia viruses expressing HLA-A2 and US11 (lanes 3-8) as indicated in the Materials and Methods section. After 4 h of incubation the cells were pulsed for 10 min and chased for 30 min. All incubations were performed at 40°C. Proteasome inhibitor ZLVS was included where indicated. HLA-A2 and US11 were immunoprecipitated sequentially, the precipitates were analysed by SDS/PAGE and PhosphorImages were generated. Bands representing HLA-A2 molecules with (HLA+CHO) or without (HLA-CHO) carbohydrate are indicated. (See next page for Figure 4 B/C)

which may form a by-pass for the inhibited proteasome.

A defective ubiquitination system results in stabilization of HLA-A2 molecules and causes HLA-A2-US11 complexes to accumulate

US11-mediated degradation of HLA-A2 was observed in ts20 cells at any temperature below the non-permissive temperature of 40°C (Figure 4A, lanes 1-9). When the experiment was performed at 40°C, the temperature at which the ubiquitin-activating enzyme E1 is inactive, HLA-A2 molecules were stabilized (Figure 4A, lanes 10-12). This result indicates that the ubiquitin system plays an essential role in US11-dependent degradation of HLA-A2. A considerable amount of US11 co-precipitated with stabilized HLA-A2 at 40°C (Figure 4A, lanes 10-12), while at the same time HLA-A2 co-precipitated with US11 (Figure

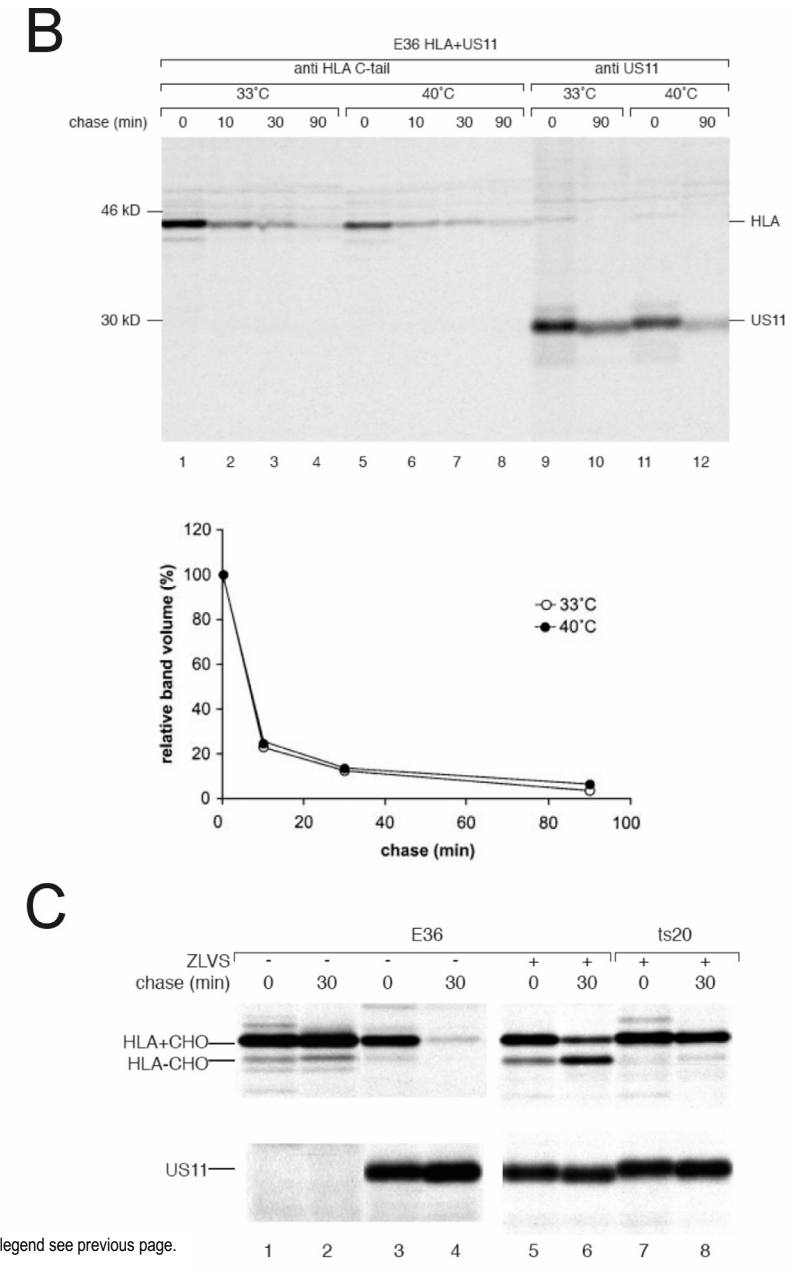

Figure 4. For legend see previous page.

4A, lanes 22-24). The identity of the co-precipitating molecules was confirmed in re-immunoprecipitation experiments (results not shown). Thus whereas binding of US11 to HLA-A2 at the permissive temperature is quickly lost due to the degradation of HLA-A2, the interaction of US11 and HLA-A2 is stabilized when ubiquitination is prohibited. In the course of the chase the mobility of US11 and HLA-A2 altered slightly, probably owing to a post-translational modification, which was not pursued further at this stage.

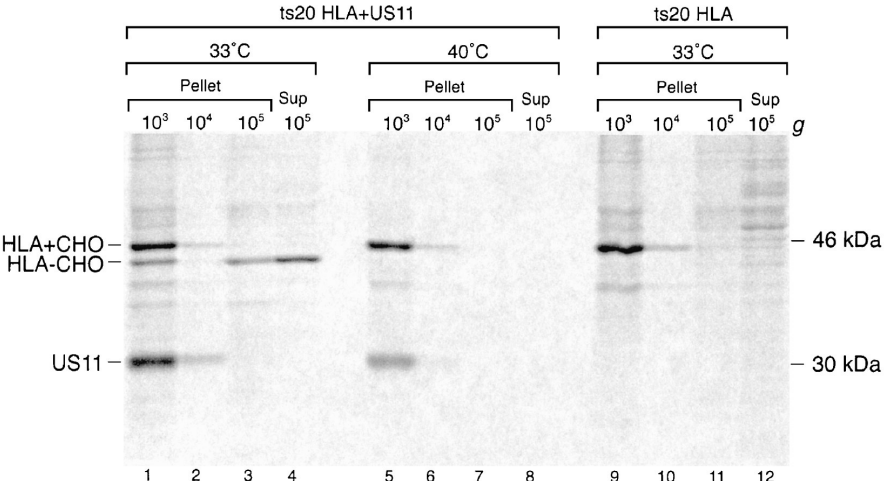

Figure 5. In the absence of ubiquitination HLA-A2 accumulates in the membrane fraction. Ts20 HLA+US11 and ts20 HLA cells were labelled with ^{35}S[methionine]/ ^{35}S[cysteine] as described in Experimental for 30 minutes in the presence of ZL_3H either at 33°C or 40°C, as indicated. The cells were homogenized with a Dounce homogenizer and subjected to repeated centrifugations at the indicated *g* forces (see also Materials and Methods section). HLA-A2 and US11 were immunoprecipitated simultaneously from each fraction using antisera against the cytoplasmic tails of MHC class I and US11. Samples were separated on a 10% SDS polyacrylamide gel. A phosphor-image was generated using a BioRad Personal Molecular Imager FX and analysed using Quantity One software.

In the E36 parental cells US11 mediated degradation of HLA-A2 was as efficient at 33°C as it was at 40°C (Figure 4B). The stabilization of HLA-A2 in ts20 cells observed at the non-permissive temperature is therefore solely due to paralysis of the ubiquitin system. In E36 cells not expressing US11, HLA-A2 was stable (Figure 4C, lanes 1 and 2). The MHC class I breakdown intermediate was observed in E36 cells expressing HLA-A2 and US11, but not in ts20 cells expressing HLA-A2 and US11, incubated at 40ºC in the presence of proteasome inhibitor ZLVS (Figure 4C, lanes 5-8). Note that in the experiment shown in Figure 4C HLA-A2 and US11 were expressed using recombinant vaccinia viruses. During later stages of vaccinia virus infection host protein synthesis is shut off, and expression of viral (trans-)genes is generally high. To avoid artefacts due to loss of stoichiometry, cells were monitored for expression of HLA and US11 only 4 h after infection. The ratio between US11 and HLA was influenced by using M.O.I. 1.5 for vvUS11 and of 1 for vvA2, to ensure sufficient US11 to degrade HLA-A2 at the permissive temperature. Degradation and stabilization of MHC class I molecules occurred in a similar fashion in stably transfected and vaccinia virus-infected cells.

HLA-A2-US11 complexes accumulate in the membrane fraction at 40°C

Is ubiquitination required only for targeting of dislocated MHC class I molecules to proteasomes, or is ubiquitination also a prerequisite for the actual dislocation of these molecules? To distinguish between these possibilities, a cell fractionation experiment was carried out (Figure 5). ts20 cells expressing HLA-A2 and US11 or HLA-A2 only were metabolically labelled for 30 min in the presence of a proteasome inhibitor. At 33°C in the double transfectant, US11 and all of the glycosylated MHC class I resided exclusively in the membrane fraction (Figure 5, 1000 *g* and 10 000 *g* pellets, lanes 1 and 2). In contrast, the deglycosylated intermediate was found predominantly in the cytosolic fraction (Figure 5, 100 000 *g* supernatant, lane 4). This pattern reflects dislocation to the cytosol and deglycosylation of MHC class I heavy chains, similar to what was shown earlier for endogenous MHC class I molecules in US11 transfected U373 cells [6]. When a similar experiment was performed on cells that were pre-incubated at 40°C, a deglycosylated intermediate was not detected. Instead, all of the HLA-A2 material was found in the membrane fraction along with US11 (Figure 5, lanes 5-8). EndoH digestion experiments showed that the HLA-A2 population rescued at 40°C

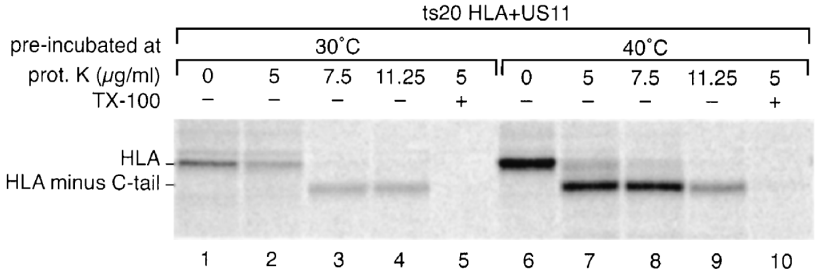

Figure 6. HLA-A2 molecules that accumulate at 40ºC do not have an altered membrane insertion. ts20 HLA and ts20 HLA+US11 cells were pre-incubated at the temperatures indicated and labelled with ^{35}S[methionine]/ ^{35}S[cysteine] for 10 min at the same temperature. The cells were semi-permeabilized and digested with proteinase K at 0ºC as was described in the Materials and Methods section at concentrations indicated. Immunoprecipitations were performed using antibodies against the ER lumenal domain of HLA-A2 heavy chains (monoclonal antibody HCA2). Samples in lane 5 and 10 were treated with 1% (v/v) triton X-100 prior to proteinase K digestion. Samples were separated on a 10% SDS polyacrylamide gel. A phosphor-image was generated using a BioRad Personal Molecular Imager FX and analysed using Quantity One software. The experiment was repeated twice with similar results. The data shown in this figure are from one such experiment.

is endoH sensitive (results not shown). Together, these data suggest that, when the ubiquitin system is disrupted, HLA-A2 molecules are not dislocated to the cytosol, but remain associated with a pre-Golgi compartment, most likely the ER. HLA-A2 from cells that do not express US11 resided in the membrane fractions, (Figure 5, lanes 9-12), as expected, since these molecules are stable during the 30 min labelling period.

At 40ºC, membrane insertion of MHC class I molecules is not altered

We next asked the question: does the membrane topology of HLA-A2 molecules change while they accumulate in the ER in the absence of ubiquitination? Previous experiments have indicated that lysines in the cytosolic tail of HLA-A2 heavy chain can be substituted without affecting dislocation and degradation [22]. The cytosolic tail is therefore not likely to be the primary target for ubiquitination. Assuming that dislocation involves ubiquitination of MHC class I molecules themselves, this would then require ER lumenal domains to become exposed to the cytosolic ubiquitin system. Such conformational changes would take place before attachment of ubiquitin, they may be independent of the ubiquitin machinery, and they may possibly be induced by the binding of US11 to the MHC class I molecules. Proteinase K digestion of MHC class I molecules in semi-permeabilized ts20 HLA+US11 cells, kept at 30ºC, results in products that lack their cytosolic C-terminal tail (Figure 6 lanes 1-4). At 40ºC, MHC class I molecules accumulate in the ER membrane, as was shown above. Figure 6 (lanes 6-9) shows that this state does not involve dramatic changes in the membrane insertion of MHC class I molecules, such as partial dislocation to the cytosol, since proteinase K digestion again resulted in removal of the cytosolic tail only.

DISCUSSION

The results described here indicate that dislocation of the MHC class I molecules across the ER membrane is fully dependent on a functional ubiquitin system. Obviously, not only dislocation requires ubiquitin, but subsequent proteasomal degradation is likely to require ubiquitination as well, since proteins are usually targeted to the proteasome via the attachment of at least four ubiquitin molecules [1,2,30]. It is not clear whether a single ubiquitination event accounts for both aspects of the degradation process, or whether several distinct ubiquitination events would be required.

The degradation process mediated by US2 and US11 now evidently includes interaction of the MHC class I molecule with the translocon channel (shown for US2 mediated degradation [7]), involvement of the ubiquitin system ([22]; and this study), dislocation to the cytosol and de-glycosylation [6] and degradation by the proteasome [6,7].

Effects of disruption of the ubiquitin system

Our experiments indicate that complexes of US11 and HLA-A2 are retained in the ER membrane when ubiquitin conjugation is blocked. Similar ER retention was observed for proteins that are degraded via endogenously initiated, quality control associated, dislocation. For example, the TCR α chain [17] and RI_{322}, a truncated ER lumenal form of ribophorin A [31], are also not dislocated when ubiquitination is

prevented. Mutation of the E2 ubiquitin carrier enzymes involved in the degradation pathway of a soluble misfolded yeast protein CPY* similarly caused the accumulation of the substrate in the ER [13]. Together, these results indicate that the ubiquitin system plays an important role in both endogenously and exogenously triggered dislocation of proteins across the ER membrane.

Possible mechanisms of ubiquitin dependent dislocation of MHC class I molecules

Although the cytoplasmic tail of the MHC class I heavy chain obviously is the part most accessible to the ubiquitination machinery, it is unlikely to be the primary target for ubiquitination, as removal of the lysine residues from the cytoplasmic tail of HLA-A2 neither prohibits its dislocation and degradation, nor its ubiquitination [22]. Interestingly, removal of all lysines from the α subunit of the T cell receptor neither affects its dislocation nor its degradation, while a functional ubiquitination system is still required for dislocation of the mutant α chain [17,32]. These results can be explained by assuming that not the degradation substrate itself, but an interacting protein is ubiquitinated, leading to *in trans* ubiquitin-mediated dislocation of the substrate. In the case of α1-antitrypsin Z it was shown that the chaperone calnexin is ubiquitinated *in trans*, leading to degradation of α1-antitrypsin Z. In the course of the process calnexin is released from the substrate and is neither dislocated nor degraded itself [33].

Since for MHC class I molecules not all of the lysines were removed, it is still a possibility that ubiquitination within ER lumenal domains of the MHC class I heavy chains mediates dislocation. Apart from lysine residues within a protein, the N-terminus can serve as a target for ubiquitination. Exclusive N-terminal ubiquitination was found for the short-lived cytosolic protein MyoD and the Epstein-Barr virus membrane protein LMP1 [34,35]. Thus, if MHC class I molecules would be ubiquitinated while still in the ER membrane, this would either involve lumenal lysine residues, or the N-terminus, which then would have to be exposed to the cytosol prior to attachment of ubiquitin. Proteinase K digestion experiments (Fig. 6) suggest that in the absence of a functional ubiquitin system the membrane topology of HLA-A2 does not drastically change. Assuming that MHC class I molecules are ubiquitinated themselves prior to dislocation, the conformational change may involve only minor exposure of HLA-A2 lumenal sequences to the cytosol, which can not be observed after proteinase K digestion. Alternatively, the topological change may not take place at the non-permissive temperature, because this by itself already requires the action of the ubiquitination machinery. Binding of MHC class I molecules to US11, which occurs without ubiquitination, apparently does not induce obvious topological changes of the MHC class I molecules.

The molecular basis of the dislocation reaction still remains obscure. It has been proposed that cytosolic chaperones such as Hsp70 and Hsp90 facilitate the actual dislocation [36,37]. The important role of ubiquitin in the dislocation process supports the suggestion that the proteasome provides the pulling force that extracts proteins from the ER membrane [38,39].

The role of US2 and US11

Inhibition of ubiquitination did not only cause a dramatic stabilization of HLA-A2, but also revealed strong binding of US11 to HLA-A2 (Figure 4A). This obviously confirms that the binding of US11 to MHC class I molecules precedes ubiquitination and that MHC class I heavy chain is not released from US11 when ubiquitination is prevented. Since US11 is not degraded and has never been found in the cytosol along with MHC class I molecules ([6] and Figure 5), the release from US11 must take place before MHC class I heavy chain is dislocated to the cytosol.

Although rather extensive research has been done on the US2 and US11 mediated degradation of MHC class I molecules, the exact functions of US2 and US11 in the dislocation process have remained elusive. Binding of US2 and US11 to MHC class I could alter the conformation of the latter such that it is recognized by the ER quality control machinery and is degraded via the constitutive dislocation pathway. US2 and US11 could also mimic components of the quality control machinery, which normally target misfolded cellular proteins for destruction. Although to date no sequence similarities have been found between US11 or US2 and any cellular protein, one could speculate that US2 and US11 themselves function as E3 enzymes, which specifically catalyse ubiquitination of MHC class I molecules, thus inducing their dislocation and degradation.

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CHAPTER 6

Human cytomegalovirus-encoded US2 and US11 target unassembled MHC class I heavy chains for degradation.

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Human cytomegalovirus-encoded US2 and US11 target unassembled MHC class I heavy chains for degradation.

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Surface MHC class I molecules serve important immune functions as ligands for both T and NK cell receptors for the elimination of infected and malignant cells. In order to reach the cell surface, MHC class I molecules have to fold properly and form trimers consisting of a heavy chain (HC), a β2-microglobulin light chain and an 8 to 10-mer peptide. A panel of ER chaperones facilitates the folding and assembly process. Incorrectly assembled or folded MHC class I HCs are detected by the ER quality control system and transported to the cytosol for degradation by proteasomes. In human cytomegalovirus-infected cells two viral proteins are synthesized, US2 and US11, which target MHC class I HCs for proteasomal degradation. It is unknown at which stage of MHC class I folding and complex formation US2 and US11 come into play. In addition, it is unclear if the disposal takes place via the same pathway through which proteins are removed that fail to pass ER quality control. In this study, we show with a β2m-deficient cell line that US2 and US11 both target unassembled HCs for degradation. This suggests that US2 and US11 both act at an early stage of MHC class I complex formation. In addition, our data indicate that US11-mediated degradation involves mechanisms that are similar to those normally used to remove terminally misfolded HCs.

MHC class I molecules are important reporters for the immune system. They display small fragments of the total cellular protein pool at the cell surface for inspection by cytoxic T cells [1]. In this way they reveal the presence of abnormal proteins expressed by malignant or infected cells. In addition, the absence or presence of MHC class I molecules can be sensed by NK cells and regulate their activation [2].

In order to reach the cell surface, MHC class I molecules have to fold properly and form a trimeric complex that consists of a heavy chain (HC; ~43 kDa), β2-microglobulin (β2m; 12 kDa) and an 8 to 10-mer peptide. The folding and assembly process occurs in an orderly fashion and is facilitated by several ER chaperones.

MHC class I HCs encode a signal peptide, which directs insertion into the ER during translation. Once in the ER, the signal sequence is cleaved off by a signal peptidase. An oligosaccharyl transferase equips the HC with an N-linked oligosaccharide at residue N86. At this stage, free HCs are found in association with the general ER chaperones immunoglobulin binding protein (BiP) [3] and calnexin (CNX), the latter of which is a membrane bound protein with lectin-like activity [4,5]. Bip binds transiently to many newly synthesized proteins and for prolonged times to misfolded proteins or unassembled subunits [6,7]. Binding of CNX is regulated by glucose trimming of nascent N-linked oligosaccharides [8]. CNX generally binds proteins with monoglucosylated (Glc1Man9-7GlnNAc2) oligosaccharides [9]. CNX and BiP predominantly associate with free MHC class I HCs and the assembly with β2m abolishes the interaction of the HC with these chaperones [10-12]. Before binding the light chain, HCs also interact with ERp57, a member of the protein disulfide isomerase (PDI) family, involved in disulfide bond oxidation, reduction and isomerization reactions [13-15]. Mature MHC class I molecules harbor three intra-molecular disulfide bridges, the formation of which is likely to be mainly assisted by ERp57.

After binding β2m, MHC class I molecules are found in association with another, soluble ER chaperone with lectin-like activity, calreticulin (CRT) [16,17]. Like CNX, CRT binds to proteins with Glc1Man9-7GlnNAc2 N-linked oligosaccharides [18,19]. These MHC class I molecules become associated with the peptide loading complex, which besides CRT includes ERp57, tapasin, and the transporter associated with antigen processing (TAP) subunits, TAP1 and TAP2. Tapasin mediates the interaction of HCs with the TAP complex [20-22]. Peptides generated from endogenous proteins by proteasomal degradation are transported from the cytosol into the ER via the TAP complex, where they can be trimmed further by amino-peptidases before

loading onto HC-β2m dimers [23,24]. Trimeric HC-β2m-peptide complexes dissociate from the loading complex and are released into the secretory pathway [25]. In contrast, incompletely assembled MHC class I HCs are recognized by the ER quality control system and are targeted for degradation [26].

During the course of HCMV infection, several viral proteins are synthesized which prevent MHC class I surface expression. These immune evasion proteins can obstruct different steps of the folding and assembly pathway of MHC class I molecules. The unique-short region 3 (US3) gene product retains MHC class I molecules in the ER and specifically affects those types of MHC class I molecules whose surface expression is tapasin-dependent [27]. US6 blocks peptide transport by TAP and thereby prevents the formation of stable trimeric MHC class I complexes [28,29]. Two other HCMV gene products, US2 and US11, both target MHC class I HCs to the cytosol for subsequent proteasomal degradation [30,31].

It is unknown if US2 and US11 make use of the regular ER quality control pathway for disposal of class I molecules. It is also unclear to what extent MHC class I molecules have to be folded and complexed with β2m and/or peptide before US2 and US11 can bind to these proteins. These aspects of US2- and US11-mediated HC degradation are investigated in the present study.

MATERIALS AND METHODS

Cell lines
Wild type FO-1 human melanoma cells [32], which have a defect in β2m gene expression, and FO-1 cells restored for β2m expression [33] were cultured in DMEM (Invitrogen, Breda, The Netherlands), supplemented with 10% FCS (Greiner bv, Alphen aan den Rijn, The Netherlands), 100 U/ml penicillin and 100 g/ml streptomycin (Invitrogen, Breda, The Netherlands). HLA class I molecules expressed by FO-1 cells were genotyped as HLA-A*2501, -B*0801, and -Cw*0701 [34].

Production of retrovirus and transduction
US2 and US11 cDNA fragments, subcloned into the pLZRS-*IRES*-EGFP vector were used for transfection of amphotropic Phoenix packaging cells to produce retrovirus, as described [35-38]. Cells were transduced with retrovirus using retronectin (Takara Shuzo, Otsu, Japan) coated dishes. Transduced cells were sorted for EGFP expression using a FACS Vantage flow cytometer.

Antibodies
The following antisera were used for immuno-precipitations: W6/32 (anti-MHC I complex; [39]), HC10 (anti-MHC I free HC's; [40]), H68.4 (transferrin receptor; Zymed Laboratories, San Francisco, CA), US2(N2) (anti-US2; [41]), and US11(N2) (anti-US11; [42]).

Metabolic labeling, cell lysis, immunoprecipitation and SDS-PAGE
Metabolic labeling, immunoprecipitations and SDS-PAGE were performed as described [43]. Where indicated, media were supplemented with the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucinal (ZL_3H). For the experiments described in Figure 1, 1mM *N*-ethylmaleimide (NEM; Sigma-Aldrich, Zwijndrecht, the Netherlands) was added to the lysis mix to prevent post-lysis formation of disulfide bonds. Peptide-N-glycosidase F (PNGase F; Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's protocol. For experiments described in figures 2 and 3, immuno-precipitations were performed on denatured lysates. Cells were lysed in a smaller volume of Nonidet-P40 lysis mix (100 µl /$5x10^6$ cells), and after centrifugation, supernatants were transferred to a new tube with 1/10 volume of 10% SDS and 1/10 volume of 0.1 M DTT. Samples were boiled for 5 min to further denature proteins. Next, the volume was increased 10 times with non-denaturing buffer (1% Triton X-100, 50 mM Tris HCl pH 7.4, 300 mM EDTA, 0.02%NaN_3) supplemented with protease inhibitors and 10 mM iodoacetic acid. Immuno-precipitates were taken up in sample buffer with (Figure 2 and 3) or without β-mercaptoethanol (Figure 1) and boiled for 5 minutes prior to loading onto 12.5 % SDS-PAGE acrylamide gels. Gels were screened with a Bio-Rad Personal Molecular Imager FX and analysed with Quantity One software.

RESULTS

It is unclear at what stage of folding and assembly of newly synthesized MHC class I HCs US2 and US11 come into play to redirect these molecules back to the cytosol for subsequent proteasomal degradation. We evaluated if US2 and US11 can target heavy chains for degradation in an early stage, namely when they are still unassembled. A β2m-negative cell line was used to address this question.

A report by Furman et al. indicated that the redox status influences degradation of class I heavy chains by US2 and US11 [44]. Mature and fully assembled MHC class I complexes contain 3 disulfide bonds: one within the β2m light chain and two within the heavy chain. The disulfide bonds in the heavy chain are located in the membrane-proximal α3 domain and in the α2 domain, the latter of which forms part of the peptide binding groove. Pulse chase experiments with wild type and mutant (C203S+C259S) HLA-A2 revealed that formation of a disulfide bond in the α3 domain of class I was essential for US2-mediated degradation, but not for degradation mediated by US11 [45]. Besides this, several studies indicated that the presence of β2m supports disulfide bond formation in MHC class I HCs [46,47]. In the absence of β2m class I HCs cycle between (fully) oxidized and reduced states [48]. In our current study we make use of the β2m-negative FO-I cell line. Before looking at the effect of US2 and US11 expression on degradation of class I heavy chains, we first investigated the differences in oxidation status of class I HCs in this cell line.

Shortly after synthesis, the majority of free class I HCs is fully oxidised in the absence or presence of β2m

We evaluated the oxidation status of MHC class I heavy chains in β2m-negative (FO-I wild type) and positive (FO-I + β2m) cell lines over time in pulse chase experiments (Figure 1). MHC class I heavy chains were recovered from NP40 lysates (supplemented with the alkylating agent NEM to prevent post lysis formation of disulfide bonds), using either HC10 or W6/32 MoAbs. Samples were separated by SDS-PAGE under non-reducing conditions. Under these circumstances, three distinct bands can be observed of which the intensity and migration patterns differ, with increasing concen-trations of the reducing agent DTT (Figure 1A). The fastest, middle and slowest migrating bands reflect fully oxidized (two disulfide bonds), partially reduced (one disulfide bond) and completely reduced HCs (no disulfide bonds), respectively.

HC10 is specific for free HC's and recognizes all HCs expressed in the β2m negative cells (Figure 1B, lanes 1-4) and only a fraction of the HC pool, likely those still unassembled, in the β2m reconstituted cells (lanes 5-8). W6/32 only recognizes HCs associated with β2m (lanes 13-16) and does not recognize HCs expressed in cells lacking β2m (Figure 1B, upper panel, lanes 9-12). To exclude a contribution of maturation of the N-linked sugar chain on the migration pattern of HC's, part of the samples were treated with PNGase F (Figure 1B, lower panel). In the presence of β2m, all W6/32-reactive material was fully oxidized (lanes 13-16) as well as the majority of

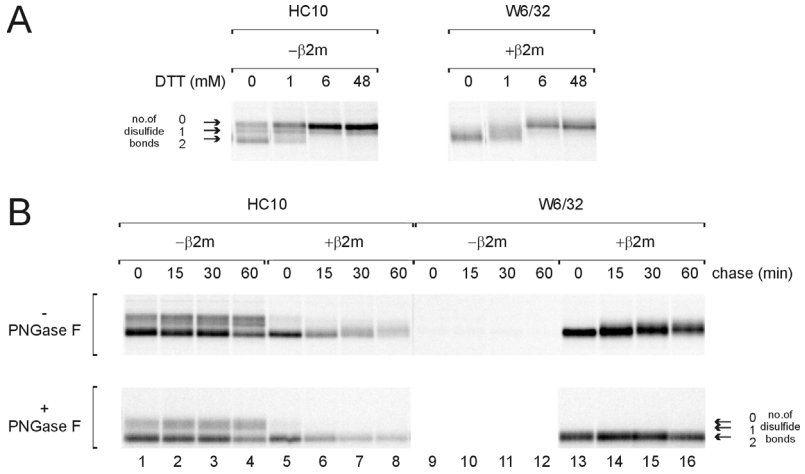

Figure 1. Shortly after synthesis, the majority of free class I HC's is fully oxidised in the presence or absence of β2m. A) FO-I cells, which have a defective β2m gene, and FO-I cells restored for β2m-expression were metabolically labeled with ^{35}S Met/Cys for 60 minutes. Cells were lysed in NP40 lysis mix (supplemented with NEM) and MHC class I HCs were recovered using MoAbs HC10 (anti-free class I HCs) or W6/32 (anti-complexed class I HCs). After immunoprecipitation, samples were split and taken up in sample buffer without or with DTT at the concentrations indicated. Samples were separated by SDS-PAGE (12.5 % gel). **B)** Cells were labeled for 5 minutes and chased for the indicated times. After immunoprecipitation, samples were split and one aliquot was treated with PNGaseF. Samples were taken up in nonreducing sample buffer. Arrows mark the migration pattern of class I HCs with 0, 1, or 2 disulfide bonds.

the HC10-reactive material (lanes 5-8). In contrast, a small amount of fully and partially reduced HC10-reactive HCs were observed in the β2m-negative cells (lanes 1-4). The relative proportion of reduced, partially reduced and oxidized HCs as compared to the total pool varied in the course of the chase in the β2m-negative cells. Right after the pulse and up to 30 minutes later the majority of HCs are fully oxidized (lanes 1-3). After a 60 minutes chase, the total pool of MHC class I is reduced. This is consistent with previous data showing degradation of free HCs in the absence of β2m [49]. At this time point, a decrease is observed in the amount of fully oxidized HCs, and a small increase in the more reduced forms, relative to the total amount of HCs (lane 4). The three distinct conformations are present in more equal amounts after 60 minutes of chase (lane 4).

Since US2 and US11 are known to act within a relatively short time window (minutes after MHC class I synthesis), they are likely to encounter fully oxidized HCs in both β2m-postive and -negative cells.

Unassembled HC's are targeted for degradation by US2 and US11.

Next, we introduced US2 and US11 into the FO-I cell lines to evaluate with pulse chase experiments if these viral proteins can target MHC class I heavy

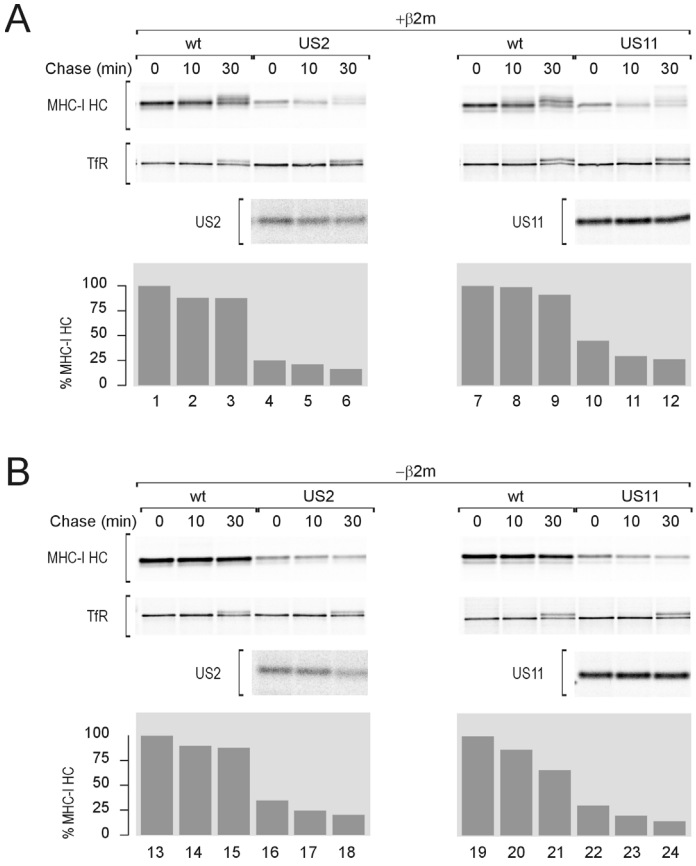

Figure 2. Unassembled HCs are targeted for degradation by US2 and US11. FO-I cells restored for β2m expression (+β2m, panel A) and wild type FO-I cells (-β2m, panel B) were transduced with wt-EGFP, US2-EGFP, or US11-EGFP-encoding retrovirus and sorted for EGFP expression. Cells were metabolically labeled with ^{35}S Met/Cys for 10 minutes and chased for the times indicated. MHC class I HCs, transferrin receptor (TfR), US2 and US11 were recovered from denatured samples, taken up in reducing sample buffer, separated by SDS-PAGE (12.5 % gel) and visualized using a phosphor-imager. The amount of precipitated MHC class I HCs, normalized on the basis of TfR levels, is displayed as a percentage of HC levels found at the onset of chase in wt-EGFP cell lines. Results are based on multiple observations, of which one representative experiment is shown here.

chains for degradation in the absence of β2m (Figure 2). After cell lysis, samples were denatured to ensure that HC10 was able to immunoprecipitate all HCs present in FO-I (+/-β2m) cell samples. Transferrin receptor immunoprecipitates are shown, as an internal control for cell labelling and sample loading. For these experiments, samples were separated by SDS-PAGE under reducing conditions. In FO-I cells reconstituted for β2m expression (Figure 2A), MHC class I heavy chains remained stable over time in the absence of viral proteins (lanes 1-3, 7-9), but are destabilized in the presence of US2 (lanes 4-6) or US11 (lanes 10-12). Note that most of the HCs have already been degraded during the ten minutes pulse, while the transferrin receptor remained stable. Figure 2B shows the effect of US2 and US11 on the stability of HCs in the absence of β2m. In the presence of US2 (lanes 16-18), less HCs could be immunoprecipitated compared to the amount recovered from US2-negative cells, while transferrin receptor levels remained the same in both cell lines (lanes 13-15). The same was observed in US11-expressing FO-I cells (compare lanes 22-24, with 19-21).

Thus, US2 and US11 can target unassembled HCs for degradation, indicating that they can act already at an early stage of MHC class I folding and complex formation.

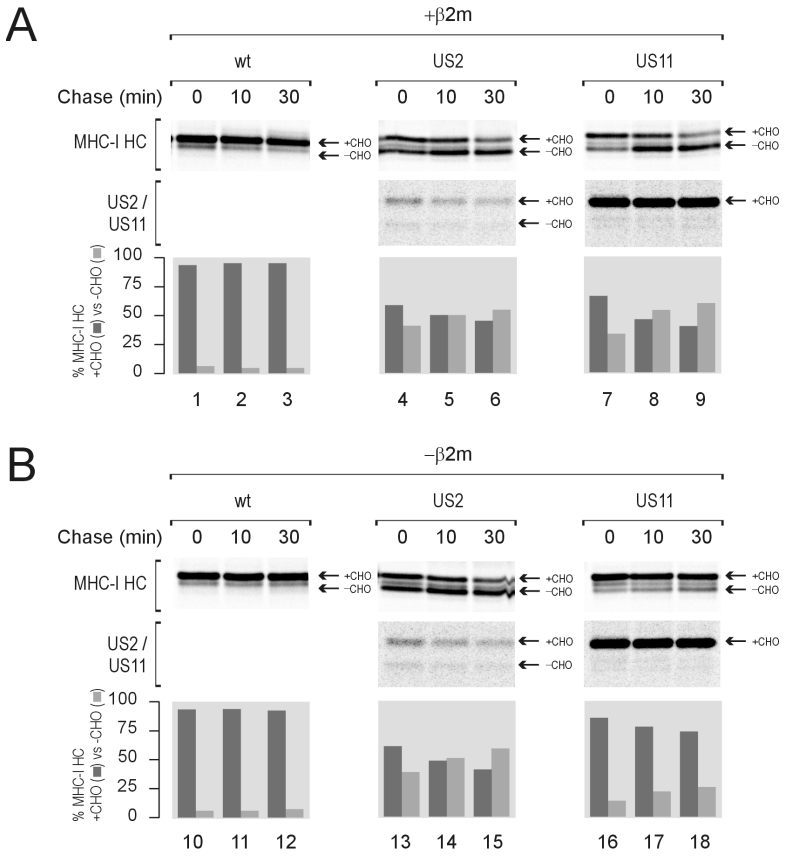

Figure 3. US11 can target HCs to the cytosol in the absence of β2m, but this action is severely compromised when proteasomal activity is blocked. FO-I cells restored for β2m expression (+β2m, panel A) and wild type FO-I cells (-β2m, panel B) were transduced with wt-EGFP, US2-EGFP, or US11-EGFP-encoding retrovirus and sorted for EGFP expression. Cells were metabolically labeled with ^{35}S Met/Cys for 10 minutes and chased for the times indicated, all in the presence of proteasome inhibitor. MHC class I HCs, US2 and US11 were recovered from denatured samples, taken up in reducing sample buffer, separated by SDS-PAGE (12.5 % gel) and visualized using a phosphor-imager. Arrows indicate migration pattern of proteins +/- glycan (CHO). The amount of MHC class I HCs +CHO or –CHO is given as a percentage of the total of MHC class I HCs (+ and – CHO) precipitated from that sample. Results are based on multiple observations, of which one representative experiment is shown here.

US11 can target HCs to the cytosol in the absence of β2m, but this action is severely compromised when proteasomal activity is blocked.

Dislocated MHC class I heavy chains can be visualized using proteasome inhibitors. Visualization is possible due to the fact that the N-linked glycan is removed from retro-translocated HCs by a cytosolic N-glycanase, before HCs are degraded by proteasomes. These breakdown intermediates are characterized by a faster migration pattern in SDS-PAGE [50,51].

To complement the data shown in Figure 2, experiments were performed in the presence of proteasome inhibitor ZL_3H (Figure 3). Figure 3A shows that in β2m expressing cells, HCs remain stable in the absence of viral proteins (lanes 1-3). In both $US2^+$ (lanes 4-6) and $US11^+$ cells (lanes 7-9), a decrease is observed in the amount of glycosylated HCs (HC+CHO) and an increase in the amount of deglycosylated breakdown intermediates (HC-CHO). The results have been quantified and displayed as graphics, with HC+CHO in dark gray and HC-CHO in light gray. Figure 3B shows the results for the β2m-negative cells. A similar conversion from glycosylated HCs to deglycosylated breakdown intermediates could be observed for the $US2^+$ cells (lanes 13-15), compared to the $\beta2m^+$, $US2^+$ cells (lanes 4-6). In contrast, only a minor fraction of HC breakdown intermediates could be observed in the $US11^+$, $\beta2m^-$ cells (lanes 16-18) as compared to the $US11^+$, $\beta2m^+$ cells (lanes 7-9) and the $US2^+$, $\beta2m^-$ cells (lanes 13-15).

These data again show that US2 can target unassembled HCs for degradation and suggest that it can do so equally well in the presence or absence of β2m, with or without proteasome inhibitor. In contrast, proteasome inhibition appears to interfere with the action of US11 in cells lacking β2m.

Inhibition of proteasome activity also delays dislocation of unassembled HCs in β2m negative cells in the absence of viral proteins.

In the absence of β2m, MHC class I HCs become a target for ER quality control mechanisms that ensure disposal of improperly assembled HCs. This has been shown using the β2m-negative Daudi cell line [52]. Pulse chase experiments showed that the dislocation

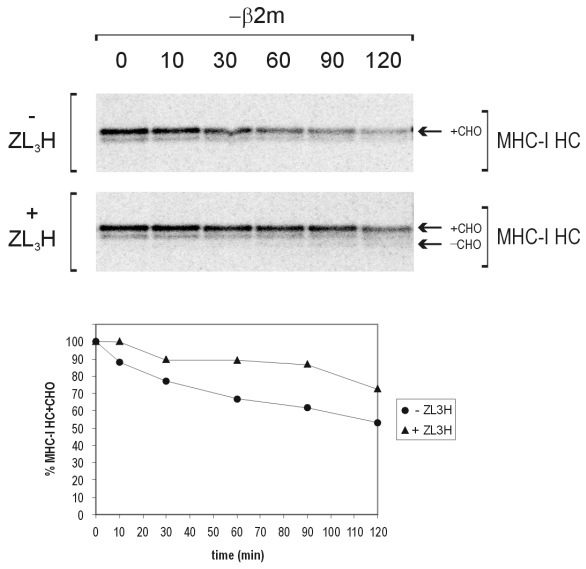

Figure 4. Inhibition of proteasome activity also delays dislocation of unassembled HCs in β2m-negative cells in the absence of viral proteins. Wild type FO-I cells (-β2m) were metabolically labeled with ^{35}S Met/Cys for 10 minutes and chased in the presence or absence of proteasome inhibitor (+/-ZL_3H) for the times indicated. MHC class I HCs were recovered from denatured samples, taken up in reducing sample buffer, separated by SDS-PAGE (12.5 % gel) and visualized using a phosphor-imager. Arrows indicate migration pattern of HCs +/- glycan (CHO). The amount of MHC class I HCs +CHO precipitated at different timepoints (relative to the total amount of HCs at the onset of the chase) is displayed graphically.

and degradation of MHC class I heavy chains takes place at a slower pace, with the first signs of dislocation showing 30 minutes after a 10 minute labelling time. We investigated if the dislocation of unassembled HCs requires proteasomal activity. For this purpose equal amounts of wild type FO-I cells were pulse labelled and chased up to 120 minutes either in the absence or presence of proteasome inhibitor (Figure 4). Equal amounts of glycosylated HCs could be precipitated at the start. Over the course of the chase, some decrease in the amount of glycosylated HCs was observed in cells treated with proteasome inhibitor, accompanied by a slight increase in the amount of deglycosylated HCs. However, this decrease of glycosylated HCs was more pronounced in the absence of proteasome inhibitor.

These results indicate that the quality control-associated dislocation of unassembled HCs is less efficient when proteasomal activity is blocked.

DISCUSSION

HCMV encodes several immune evasion proteins that prevent MHC class I surface expression. These viral gene products can obstruct different steps of the folding and assembly pathway of MHC class I molecules. We investigated at what stage of the assembly process MHC class I HCs are redirected to the cytosol by US2 and US11 for proteasomal degradation.
Previous observations suggest that US2 prefers properly folded and assembled HCs as target; it can be found in association with assembled MHC class I molecules (indicated by its co-precipitation with the conformation-dependent anti-MHC I complex antibody W6/32) [53]. In addition, US2 co-crystallized with class I HC-β2m-peptide complexes [54].

In this study, we evaluated in pulse chase experiments if US2 and US11 are capable of targeting free HCs for degradation. For this purpose, we used a human melanoma cell line (FO-I), which does not express β2m [55]. β2m-reconstituted FO-I cells served as a control. Surprisingly, US2 as well as US11 could target free HCs for degradation. Moreover, this occurred with an efficiency that appeared to be similar to that observed in cells expressing β2m (Figure 2). This shows that US2 and US11 can both act at early stages of MHC class I assembly.

These data are in disagreement with a previous report, which suggested that US2-mediated dislocation of MHC class I HCs requires assembly with β2m [56]. This conclusion was based on experiments performed with a human astrocytoma cell line (U373-GM) in which RNA interference (RNAi) was used to knock down β2m-expression. US2-mediated dislocation of class I HCs was much less efficient in these β2m-knock out cells than in wild type cells, as indicated by a slower conversion of glycosylated to deglycosylated HCs in the presence of proteasome inhibitor. Our data suggest that another factor than the absence of β2m may be responsible for the slowed down US2-mediated retro-transport of HCs in these U373-GM β2m-knock out cells. In our experiments, we could see similar amounts of deglycosylated breakdown inter-mediates for both FO-I and β2m-reconstituted FO-I cells (expressing similar amounts of US2), when proteasome inhibitor was included (Figure 3). It may be that cell type specific factors render FO-I cells more suitable to facilitate US2-mediated degradation of free HCs than U373-MG cells. Alternatively, the RNAi construct used may, besides knocking down β2m-expression, also influence the expression of other factors important for the efficiency of the dislocation process.

We showed that HCs do not require assembly with β2m in order to become targets for US11 either. The efficiency of HC degradation in the presence of US11 is similar in $\beta2m^+$ and $\beta2m^-$ FO-I cells (Figure 2). Interestingly, the inclusion of proteasome inhibitor seriously obstructed the dislocation efficiency of HCs, but only for $US11^+$, $\beta2m^-$ cells (Figure 3). This was not observed in $US2^+$ $\beta2m^+$, $US2^+$, $\beta2m^-$, nor $US11^+$ $\beta2m^+$ cells. Why was this obstruction for dislocation seen only in the presence of proteasome inhibitor, and why only in cells lacking β2m-expression? And why is this observed in US11-positive cells, but not in cells expressing US2?

In the absence of US2 or US11, incompletely folded or assembled MHC class I molecules are also removed from the ER and transported to the cytosol where they are degraded by proteasomes [57]. We showed that the dislocation of unassembled HCs in FO-I cells occurred with lower efficiency in the presence of proteasome inhibitors, in the absence of US11 (Figure 4). A similar observation has been reported for β2m-negative Daudi cells [58]. Interestingly, treatment with chemicals that interfere with disulfide

bond formation (diamide, NEM), also abrogated dislocation of HCs in Daudi cells [59].

MHC class I HCs expressed in cell lines with or without β2m are known to differ for their interaction with ER chaperones. Analysis of human β2m-deficient cells has shown that the light chain is required for correct folding, binding to calreticulin and TAP, peptide loading, intracellular transport, and cell surface expression of HLA class I heavy chains [60,61]. In the absence of β2m, HCs do not enter the secretory pathway, but remain associated for a prolonged time with BiP and calnexin [62,63]. The exact mechanism by which misfolded and unassembled molecules are finally removed from the ER remains elusive, but there are indications that these ER chaperones may play a role in this process.

BiP is known to retain many misfolded proteins in the ER [64], including unassembled MHC class I HC's [65]. Studies with mutant Kar2p (the yeast homologue of BiP) and mutant glycoprotein (CPY*), have shown an association between the ATPase activity of Kar2p with release of malfolded proteins into the cytosol [66]. In another report, studying the release from BiP of a soluble nonglycosylated protein, unassembled Ig L chain, and its retro-translocation out of the ER, the dislocation seemed to be tightly coupled to proteasome activity [67].

Calnexin, a lectin chaperone, accompanies many glycoproteins during their folding [68,69]. It can also contribute to oxidative folding, as it acts in conjunction with the oxidoreductase ERp57 [70]. We showed that in the absence of β2m, the majority of HCs is fully oxidized shortly after synthesis. When these HCs are followed in time, a larger amount, relative to the total HC pool at that time, is found in a partially or completely reduced state (Figure 1). The total amount of HCs gradually becomes less, as unassembled HCs are targeted for degradation (Hughes, Hammond, and Cresswell 1997, and Figure 4). This conversion of HCs to a reduced state may be a prerequisite for efficient dislocation. The finding that diamide and NEM abrogated dislocation in the β2m-negative Daudi cell line supports an influence of protein redox status on dislocation [71]. There are indications that proteasome inhibitors may interfere with CNX/oxido-reductase interactions [72,73]. In cells treated with lactacystin, a redistribution of ER chaperones was observed: upon proteasome inhibition, CNX, CRT, and ER degradation substrates (but not BiP, PDI, glucosyltransferase, ERp57) accumulated in a pericentriolar quality control compartment derived from the ER [74,75].

The fact that we found an abrogation of US11-mediated dislocation, in the presence of proteasome inhibitor only and exclusively in cells lacking β2m-expression may imply that US11 uses partially similar mechanisms for discarding HCs as the endogenous pathway used by FO-I cells to dispose of unassembled class I molecules (Figure 3).

All in all, we conclude that US2 and US11 can act on MHC class I molecules at an early stage of folding and assembly. In addition, our data indicate a link between the endogenous pathway for disposal of terminally misfolded proteins and US11-mediated degradation of MHC class I HCs. More research will be required to unravel the exact partners that link up these processes.

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CHAPTER 7

Summary and Discussion

Summary and discussion

HCMV encodes several proteins that interfere with cross talk between infected host cells and immune effector cells. The viral gene products can reduce surface levels of antigen presenting MHC class I molecules. Several aspects may influence the degree of MHC class I down-modulation by HCMV, and thereby the success of immune escape by the virus.

First of all, the success of immune evasion is likely to be influenced by the specificity of MHC class I down-modulation, as a complete down-regulation of MHC class I surface expression would render cells susceptible to NK cell lysis. A selective down-modulation of only those alleles that present viral antigens to cytotoxic T cells (mainly HLA-A and –B locus products) could ensure both T and NK cell escape.

Several HCMV-encoded proteins contribute to the down-regulation of MHC class I molecules in the course of infection. They differ from each other in mechanism and selectivity in down-modulatory effect on the different MHC class I locus products. US3, an immediate early HCMV gene product, mainly affects surface expression of tapasin-dependent MHC class I alleles [1]. By blocking TAP, US6 prevents peptide transport into the ER and subsequent peptide loading at early and late times post infection [2,3]. This affects surface expression of all MHC class I alleles. In spite of this, surface expression of HLA-E molecules can be preserved by supplying it with a TAP-independent peptide [4,5]. US2 and US11, expressed at early times post infection, target newly synthesized MHC class I molecules for degradation [6,7]. The effect of these viral gene products on surface expression of different subsets of MHC class I molecules will be discussed below.

In the studies described in Chapters 2,3 and 4, we aimed to clarify how, and to what extent, US2 and US11 can contribute to the efficiency and specificity of MHC class I down-regulation. Previous studies had mainly focussed on the mechanism of interference with antigen presentation [6,8-11]. With the cell lines and antibodies used in these studies, it is difficult to deduce effects on individual MHC class I locus products. To enable detection of the effects of US2/US11 on individual MHC class I alleles, we introduced different class I heavy chain constructs into a murine cell line which co-expresses human β2m. We evaluated the effect on MHC class I surface expression using flow cytometry, as this is most relevant with respect to NK and T cell receptor interactions. Moreover, MHC class I molecules that appear stable in the presence of US2 and US11 in pulse-chase experiments, could nevertheless be withheld from the surface via other mechanisms than degradation. As shown in Chapter 2, US11 can cause ER retention of MHC class I molecules that are not targeted for degradation.

Flow cytometry studies showed that US2 selectively down-modulates HLA-A2, -B27, and HLA-G, but not HLA-B7, HLA-Cw3, or HLA-E (Chapter 3). Similar studies with US11 showed differences in selectivity, with HLA-A2, -B7, -B27, -Cw3 being sensitive, and HLA-G and -E being insensitive to US11-mediated down-regulation (Chapters 2 and 4). These flow cytometry studies complemented and refined previously performed pulse chase experiments evaluating stability changes of MHC class I molecules in the presence of US2 or US11 [9,12,13], as well as previously performed *in vitro* binding studies performed with US2 and various MHC class I alleles [14]. Not all HLA-C molecules are insensitive to US2, as HLA-Cw7 and -Cw2 were down-regulated in its presence [15]; our own unpublished results).

It is clear that there are sensitivity differences between the various MHC class I locus products, but also between gene products of a single locus subgroup, for down-modulation by US2 and US11. Our next step was to unravel the determinants for this selectivity. This would allow sensitivity predictions for a larger variety of MHC class I molecules.

Selectivity of US2-mediated degradation of MHC class I molecules

Based on crystal structure data of HLA-A2-β2m-US2 complexes and sequence alignments for the region of class I implicated in US2 binding, we hypothesized that allelic variation in the α2/α3 ER-lumenal region might explain the resistance of HLA-B7, -Cw3 and -E as opposed to the sensitivity of HLA-A2, -B27 and -G [16,17]. In Chapter 4, we investigated whether we could convert the resistance of HLA-B7 and HLA-E by replacing those residues that are assumed to prohibit an interaction with US2, with the corresponding residues found in US2-sensitive molecules. The results from experiments with HLA-B7 $^{ET(L)Q}$ and HLA-E ^{QRTD} mutants showed that residues in this region are

indeed important sensitivity determinants. Even a single mutation in HLA-E (H181R) significantly altered the sensitivity to down-modulation by US2.

HLA-Cw3 differs at positions 183 (E) and 184 (H) from many US2-sensitive class I molecules (carrying D and A/P at positions 183 and 184, respectively). However, this variation appeared not to be responsible for its resistance, as US2-sensitive HLA-Cw2 and -Cw7 also carry E183 and H184 residues. Nonetheless, sequence variation at this site may still affect the efficiency of down-modulation, as US2-resistant HLA-Cw3 molecules became somewhat more sensitive when E183D, H184P mutations were introduced (unpublished results). However, the presence of a positively charged lysine residue at position 173 is the most likely explanation for the resistance to US2-mediated down-regulation of HLA-Cw3, since US2-sensitive alleles, including the majority of HLA-C alleles, have a negatively charged glutamic acid at position 173.

The crystal structure data showed which region of the ER-lumenal part of HLA-A2 was implicated in US2 binding, but did not provide insights into possible interactions between transmembrane and cytosolic regions of class I heavy chains and the US proteins. Sequence alignments and studies using chimeras derived from US2-sensitive and -insensitive alleles make it unlikely that sequence variation outside the ER-lumenal part of HLA contributes to sensitivity differences to US2-mediated down-regulation (Chapter 4).

Sequence alignments of the region implicated in US2-binding show that HLA-A2, -B27, -E and -G are identical to their locus subgroup consensus sequences for this region. This makes it likely that the majority of HLA-A, B, -E and -G will be down-modulated in the presence of US2. However, not all HLA-B molecules are affected by US2, as exemplified by HLA-B7. Those residues that were shown to be responsible for the resistance of HLA-B7 can also be found in several other HLA-B alleles, including HLA-B8, -B40, -B41, -B42, and –B48. These HLA-B alleles, which are relatively common (found in 25-30% of the Caucasion population; F. Claas, unpublished observation), are likely to be insensitive to US2-mediated down-regulation.

We did not find any indication that sequence variation in the domains located outside the ER-lumenal region contributes to sensitivity differences between class I locus products for US2 (Chapter 4). The pulse chase experiments with HLA-G showed that class I heavy chains do not need to be equipped with a full-length tail in order to be a target for US2-mediated degradation (Chapter 4). However, heavy chains comprised of ER-lumenal regions only, are inadequate targets for US2, as illustrated by the resistance of the soluble HLA-G1 isoform (Chapter 4).

Requirements for US11-mediated degradation of MHC class I

For US11, the only MHC class I locus products that appear to be completely insensitive to down-modulation are HLA-G and HLA-E. Interestingly, all that is required to confer sensitivity to these two MHC class I locus products, is an extension of their cytoplasmic tail. HLA-G molecules naturally have a tail of 6 residues, and an extension of this tail with 27 residues derived from the tail of HLA-A2 resulted in a very efficient degradation of these mutants in US11-positive cells (Chapter 2). Surprisingly, a similar HLA-G mutant with 25 instead of 27 extra tail residues did not show this result. Apparently, the C-terminal lysine and valine residues were essential for degradation. HLA-E has a cytoplasmic tail that lacks only 4 residues compared to HLA-A molecules. HLA-E required only 2 extra residues (lysine and valine) to become sensitive to US11 (Chapter 4). This clearly shows that length and amino acid composition of the tail can be important determinants for sensitivity differences between MHC class I locus products.

However, lysine and valine residues are not the only requirements, nor are they essential in all cases. HLA-B alleles do not encode for C-terminal lysine and valine residues, but are nevertheless down-modulated by US11. Likewise, a mutant HLA-A2 with a tail as long as that of HLA-B molecules (HLA-A2delCKV) and HLA-A2 with the tail of HLA-E were still down-regulated by US11 (Chapter 4). These KV residues may, however, determine the effectiveness or threshold for down-modulation, as the mutant constructs that lacked these residues appeared to require higher levels of US11 than their wild type counterparts for a similar down-regulatory effect. Besides cytosolic residues, other regions of class I also play a role. Studies with HLA-A2/G and HLA-A2/E chimeras showed that the α1-α3 ER-lumenal residues influence sensitivity to US11 (Chapters 2 and 4). A crystal structure of complexed US11/MHC class I would be very helpful to narrow down the exact region and residues of class I heavy chains that are involved in US11 binding. The studies with HLA-A2/G

and HLA-A2/E chimeras did not reveal a role for the connecting peptide or trans-membrane regions in locus product sensitivity differences (Chapters 2 and 4).

The results from Chapters 2 and 4 give rise to the following model: In principle, all MHC class I cytoplasmic domains, with the exception of HLA-G, have an amino acid composition that can make them targets for US11-mediated degradation. However, a minimum of 29 class I tail residues has to go with either a favourable ER-lumenal region, or with lysine and valine tail residues in order to evoke sufficient down-modulation by US11. A favourable ER-lumenal region may bypass the function of the KV residues through a prolonged and/or stronger interaction with US11, thereby increasing the chances of getting dislocated to the cytosol for subsequent degradation by proteasomes.

The function of the KV, as well as of the other tail residues is still unclear. Addition of ubiquitin moieties to degradation substrates may provide a handle for extraction of proteins across the ER membrane. Enhancement of MHC class I down-regulation by KV residues may rely on the lysine, functioning as a potential ubiquitination site.

Using a cell line carrying a temperature-dependent defect in the E1 ubiquitin-activating enzyme, we have shown that ubiquitination is essential for US11-mediated dislocation of MHC class I molecules (Chapter 5). At the non-permissive temperature, MHC class I molecules remained in the ER lumen. These data clearly showed the importance of ubiquitination for the dislocation of MHC class I molecules induced by US11. However, it remains unclear which protein(s) need to be ubiquitinated to trigger the dislocation process.

A study by Shamu et al. showed that lysine residues in the tail of HLA-A2 are not essential for US11-mediated degradation. However, elimination of a mutant HLA-A2 lacking lysine residues in the cytoplasmic tail appeared to be retarded compared to wild type HLA-A2 [18].

Alternatively, the KV residues may merely facilitate access of components of the dislocation and degradation machinery to essential residues residing within another region of the tail. Phosphorylation can be an important signal for docking of E3 ligases, which in turn can ubiquitinate their substrates [19]. The tail of MHC class I molecules encodes several potential as well as acknowledged phosphorylation sites [20-25]. Substitutions of the known Y321 and S335 phosphorylation sites did not alter the sensitivity to US11-associated degradation (Chapter 2 and un-published results). More research will be required to unravel why the tail is essential for US11-mediated dislocation of MHC class I molecules.

Clearly, sequence variation in MHC class I molecules is an important determinant for their sensitivity to down-modulation by several of the US proteins. At present, it is unknown whether the sequence of the US region of laboratory HCMV strains differs from that in strains circulating in the population.

Besides the specificity, the degree of MHC class I down-regulation is likely important for a successful immune escape of HCMV. *In vivo*, MHC class I expression is upregulated in the presence of cytokines. When the expression of MHC class I was elevated *in vitro*, higher levels of US proteins were required for a sufficient down-modulatory effect (Chapter 3). Also, cells infected with a higher HCMV copy number show a higher degree of MHC class I down-regulation, compared to cells infected with lower copy numbers [26].

Why would HCMV encode for so many proteins with the same purpose, namely to prevent immune detection through down-modulation of MHC class I molecules? In part, this may reflect the fact that these proteins show temporal expression patterns. Moreover, expression of more than one immune evasion gene at a given time during infection could ensure a more efficient down-modulation of one particular MHC class I locus product. A concerted action of US proteins could also affect a broader range of MHC class I locus products. Moreover, by using different mechanisms, an exhaustion of one particular route can be prevented.

Two of the HCMV-encoded proteins, US2 and US11, show similar temporal expression patterns, and seem to act very similarly. We evaluated the need for expression of both these proteins.

Differences between US2 and US11

Whereas specificity of US2-mediated down-modulation seems to rely mostly on a region at the junction of the α2/α3 domain, our data indicate that the conditions are different and more complicated for US11. Although ER-lumenal residues of HLA class I

do play a role, replacement of residues LHLE in the HLA-E α2/3 region by QRTD did not affect its sensitivity to US11 (unpublished results). Association of US2 and US11 with distinct domains of class I molecules may contribute to a broader defense of HCMV. Together, US2 and US11 could attack a wider variety of MHC I molecules.

Whereas US2 does not require MHC class I tail residues, US11-mediated down-modulation depends largely on this region. Interestingly, the opposite has been observed for US2 and US11 deletion mutants. When US2 was deprived of its tail residues it was no longer functional, while tailless US11 could still target class I molecules for degradation [27,28].

Whereas US2 accompanies its targets to the cytosol, US11 seems to remain behind in the ER [7,29]. This is accompanied by a prolonged stability of US11, compared to US2. In the presence of proteasome inhibitors, a deglycosylated cytosolic intermediate can be observed for US2.

At present, several proteins and protein complexes have been identified that could form a link between the dislocation process and proteasomal degradation of ER lumenal proteins in general, and MHC class I molecules in particular. The cytosolic ATPase p97 (also called VCP or Cdc48 in yeast) and its cofactor, a dimer of Ufd1 and Npl14, have been implicated in the retrograde transport of ER substrates [30,31].

Recently, a new protein has been identified, which interacts with the transmembrane region of US11 [32,33]. This protein, named Derlin-1, is a homologue of yeast Der1, which was shown to be required for degradation of misfolded lumenal ER proteins [34]. Derlin-1 is predicted to have 4 transmembrane regions and cytosolic N- and C-termini. Derlin-1 is essential for US11-mediated dislocation of class I molecules, but not for dislocation of class I heavy chains induced by US2. Interestingly, dislocation of US2 itself is derlin-1 dependent [35].

Derlin-1 interacts with another protein called VCP-interacting membrane protein (VIMP). VIMP is predicted to have a single transmembrane spanning domain, a short ER lumenal domain and a relatively long (~132 aa) cytosolic region. The cytoplasmic region of VIMP links the p97/VCP complex to the ER membrane [36]. The p97-Ufd1-Npl4 complex can recognize poly-ubiquitin chains, and ATP hydrolysis by p97 could provide the driving force to finally pull the substrate into the cytosol [37].

It is unknown if other ER-localized proteins facilitate the disposal of US2 and US11 targets. It is tempting to speculate on US2 and US11 making use of proteins normally engaged in quality control and disposal of misfolded ER proteins. It is known that in the absence of other complex components, e.g. the β2m light chain or peptides, free class I heavy chains cannot meet the quality standards in the ER and are targeted for degradation [38]. Different ER chaperones are assisting different stages of the folding and assembly of MHC class I molecules. In Chapter 6, we investigated if US2 and US11 can act at relatively early stages of MHC class I folding and complex formation, i.e. before association with β2m. We found that both US2 and US11 can target free HCs for degradation, as evaluated in a β2m-negative cell line. They did so equally well, in cells lacking β2m and in β2m-reconstituted cells.

Interestingly, the presence of the proteasome inhibitor ZL_3H severely decreased the efficiency of heavy chain dislocation, but only when mediated by US11 and only in cells lacking β2m. This was not observed with a similar experimental set-up including US2, nor in the absence of proteasome inhibitor, nor in $US11^+$, $\beta2m^+$ cells supplemented with ZL_3H.

The ER chaperones that are generally found in association with free class I HCs are BiP, calnexin, and the oxidoreductase ERp57. BiP is known to retain many misfolded proteins in the ER [39]. Studies with mutant Kar2p (the yeast homologue of BiP), have shown an association between the ATPase activity of Kar2p with release of malfolded proteins into the cytosol [40]. In another report, in which the release of misfolded immunoglobulin light chains from BiP was studied, the dislocation seemed to be tightly coupled to proteasome activity [41].

Calnexin serves as a chaperone for many glycoproteins [42,43]. It may also contribute to oxidative folding, as it acts in conjunction with the oxido-reductase ERp57 [44]. It is possible that class I heavy chains require a more reduced oxidation status for efficient dislocation. There are indications that proteasome inhibitors may interfere with calnexin / oxidoreductase interactions [45,46]. In cells treated with lactacystin, a redistribution of ER chaperones was observed. Upon proteasome inhibition, CNX, CRT, and ER degradation substrates (but not BiP, PDI,

glucosyltransferase, or ERp57) accumulated in a pericentriolar quality control compartment derived from the ER [45,46].

The fact that we found an abrogation of US11-mediated dislocation in the presence of proteasome inhibitor and exclusively in cells lacking β2m-expression may imply that US11 uses partially similar mechanisms for discarding HCs as the endogenous pathway used by FO-I cells to dispose of unassembled class I molecules. It also implies that it uses a mechanism distinct from that used by US2. More research will be required to elucidate the exact pathways of protein disposal.

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Nederlandse samenvatting

Het menselijk lichaam wordt voortdurend blootgesteld aan allerlei virussen. Er bestaan meerdere afweermechanismen die bescherming kunnen bieden tegen ongewenste indringers. De eerste afweerlinie wordt gevormd door barrières als huid, slijmvliezen en een zure omgeving (zoals in de maag). Als virusdeeltjes de gastheer toch weten binnen te dringen, treden meer geavanceerde afweermechanismen in werking die de detectie en eliminatie van het virus proberen te bewerkstelligen.

Een virus kan zichzelf niet vermenigvuldigen en is afhankelijk van zijn gastheer voor de productie van nageslacht. Het zal een gastheercel moeten binnendringen om daar nieuwe virusdeeltjes te laten maken. Voordat een virusdeeltje een cel heeft geïnfecteerd, kan het echter worden ontdekt door een specifieke tak van het immuunsysteem, namelijk B cellen. Deze B cellen produceren antilichamen die structuren aan de buitenkant van het virus kunnen herkennen. Door aan het virusdeeltje te binden kunnen antilichamen het virus markeren om te worden opgeruimd door het immuunsysteem. De antlichaamproducerende B cellen ontwikkelen ook een geheugen zodat ze bij een volgende ontmoeting met een zelfde virus sneller kunnen reageren.

Als een virusdeeltje toch een cel is binnengedrongen, komt een nieuwe tak van het immuunsysteem in werking, namelijk de T cellen. Deze afweercellen kunnen lichaamsvreemde structuren (antigenen) aan de buitenkant van een geïnfecteerde cel detecteren. Geïnfecteerde cellen dragen kleine fragmentjes van het virus op het celoppervlak. Deze antigenen liggen ingebed in moleculen die worden gecodeerd door het zogeheten Major Histocompatibility Complex (MHC).

MHC moleculen zijn onder te verdelen in twee klassen, die elk op verschillende celtypen voorkomen en ook gebruik maken van andere bronnen voor hun antigeen-fragmenten. MHC klasse I moleculen zijn op nagenoeg alle celtypen te vinden en presenteren over het algemeen fragmenten van eiwitten die in de eigen cel zijn gemaakt. MHC klasse II moleculen komen alleen voor op speciale antigeenpresenterende celtypen (B-cellen, macrofagen en dendritische cellen) en binden voornamelijk fragmenten die van buitenaf zijn opgenomen.

Cytotoxische T cellen kunnen de lichaamsvreemde virus fragmentjes ontdekken op MHC klasse I moleculen van geïnfecteerde cellen en vervolgens overgaan tot de aanval om deze cellen te doden. Zij doen dit met hulp van een andere categorie T cellen, zogenaamde helper T cellen, die gespecialiseerd zijn in de herkenning van antigenen op MHC klasse II moleculen. Als de aanwezigheid van een virus op deze manier is gedetecteerd en de geïnfecteerde cellen zijn opgeruimd, wordt daarmee gelijk de productie van nieuwe virusdeeltjes stop gezet.

Het virus waarover dit proefschrift gaat, is het humane cytomegalovirus (HCMV). Een groot deel van de wereldbevolking is met dit virus geïnfecteerd. Het vormt geen bedreiging voor gezonde mensen, maar mensen met een onderontwikkeld of verzwakt immuunsysteem (oa. AIDS patiënten of mensen die een transplantatie hebben ondergaan en immuniteit-onderdrukkende middelen krijgen toegediend) kunnen er ernstig ziek van worden en er zelfs door overlijden. Het humaan cytomegalovirus kan ook aangeboren afwijkingen veroorzaken als een moeder tijdens de zwangerschap voor het eerst een infectie met het virus oploopt. Zelfs een goedwerkend immuunsysteem is niet in staat het virus te elimineren, waardoor de gastheer de rest van het leven drager blijft van dit virus. Het virus verkeert overigens het merendeel van de tijd slechts in een soort sluimertoestand (latente infectie) en wordt maar nu en dan actief.

Tijdens de actieve infectiefase gebruikt het virus een aantal mechanismen waarmee het tracht te ontkomen aan detectie door het immuunsysteem van zijn gastheer. Het zorgt er onder andere voor dat er in geïnfecteerde cellen virale eiwitten worden aangemaakt die verhinderen dat MHC klasse I moleculen virusfragmentjes naar het celoppervlak kunnen brengen. Tijdens hun inspectieronde zullen T cellen op deze manier niet door hebben dat er een virus in de cel aanwezig is en de cel met rust laten. Echter, cellen die helemaal geen MHC klasse I moleculen meer op hun celoppervlak hebben zijn ook verdacht. Zogeheten Natural Killer (NK) cellen kunnen zo'n cel alsnog doden.

Er zijn meerdere soorten MHC klasse I moleculen, waaronder HLA-A, -B, -C, -E, en -G. Van HLA-A, -B en -C kunnen vele varianten voorkomen in de populatie, terwijl HLA-G en-E moleculen in bijna ieder individu hetzelfde zijn. De HLA-A, -B, -C, -E, en -G subgroepen verschillen ook in de mate waarin, en het

celtype waarop ze voorkomen, evenals in hun vermogen om een (grote) variatie aan antigeen fragmentjes te presenteren. Met name de HLA-A en -B moleculen zijn belangrijk voor de presentatie van lichaamsvreemde antigenen aan T cellen. De andere HLA moleculen zijn tot op zekere hoogte ook in staat om antigenen te presenteren, maar spelen vermoedelijk een belangrijkere rol bij het in toom houden van de NK cellen.
Nagenoeg alle cellen hebben HLA-A, -B, -C, en -E moleculen op hun celoppervlak. HLA-G komt voornamelijk voor op cellen gelegen op het grensvlak tussen moeder en foetus, in de placenta. Op deze cellen bevinden zich naast HLA-G ook HLA-C en -E, maar geen HLA-A of -B moleculen. Aangezien de HLA-A en -B moleculen in de foetale cellen voor een deel anders zullen zijn dan die van de moeder, kan hun afwezigheid hier beschermen tegen eventuele aanvallen van maternale immuuncellen.

Voordat een MHC klasse I molecuul zich vanaf het celoppervlak kan presenteren aan immuuncellen, is er eerst nog een heel assemblage- en keuringsproces aan voorafgegaan. In de celvloeistof (het cytosol) ligt een netwerk van membranen, het endoplasmatisch reticulum (ER), dat betrokken is bij de bouw en het transport van eiwitten. Nieuwe MHC klasse I moleculen worden vanuit het cytosol in het ER aangeleverd in een soort bouwpakket bestaande uit drie losse onderdelen. Het grootste onderdeel (de zware keten, HLA-"X") zit in het membraan verankerd, met een klein staartje in het cytosol en met een groter gedeelte in het ER. Aan dit ER-deel wordt allereerst een lichte keten (β2m) bevestigd en vervolgens een antigeen fragment (peptide). Deze assemblage wordt ondersteund door verschillende chaperonne-eiwitten die ervoor zorgen dat de verschillende losse onderdelen de juiste conformatie kunnen aannemen en nauwkeurig in elkaar passen. Daarnaast vinden er ook kwaliteitscontroles plaats. Misvormde en ongeassembleerde MHC klasse I moleculen worden eruit gepikt en vernietigd in het cytosol. Alleen 'goedgekeurde' MHC klasse I moleculen worden uiteindelijk op transport gezet naar het celoppervlak.

De studies in dit proefschrift zijn gericht op de eiwitten US2 en US11, die tijdens een infectie met humaan cytomegalovirus worden gemaakt. Deze twee viruseiwitten bevinden zich in het endoplasmatisch reticulum en binden daar aan nieuw aangemaakte MHC klasse I moleculen. US2 en US11 zorgen ervoor dat deze MHC moleculen voor vernietiging naar het cytosol worden gedirigeerd.

In **Hoofdstukken 2, 3 en 4** van dit proefschrift is beschreven welke typen HLA moleculen doelwit zijn van de acties van US2 en US11. Hiervoor is gekeken naar het effect van deze virale eiwitten op een aantal afzonderlijke MHC klasse I moleculen. De resultaten laten zien dat er veel minder HLA-A2, HLA-B27 en HLA-G moleculen op het celoppervlak voorkomen in US2-positieve cellen, terwijl het aantal HLA-B7, HLA-Cw3 en HLA-E moleculen gelijk blijft. In aanwezigheid van US11 daarentegen, is er geen effect te zien op HLA-G of HLA-E, maar worden er veel minder HLA-B moleculen (B7 en B27) en HLA-Cw3 en HLA-A2 moleculen op het celoppervlak gevonden. Deze studies geven aan dat US2 en US11 ieder een verschillend pakket van MHC klasse I moleculen als doelwit hebben. Ook is duidelijk geworden dat een deel van de MHC klasse I moleculen aan de acties van US2 en US11 kunnen ontsnappen. Dit laatste zou wel eens voordelig kunnen zijn voor het virus, omdat het hierdoor ook een aanval van Natural Killer cellen kan voorkomen.

Van HLA-G en HLA-E zijn heel weinig varianten te vinden in de populatie, van HLA-C redelijk veel en van HLA-A en HLA-B nog veel meer. Een ander doel van de experimenten was erop gericht een indruk te krijgen hoe representatief de geteste HLA moleculen nu eigenlijk zijn voor hun subgroep. Om voorspellingen te kunnen doen over de gevoeligheid van andere HLA moleculen voor US2 en US11, is het belangrijk om meer te weten over de interactie van deze virale eiwitten met hun doelwit. Daarom is er gekeken welke domeinen van MHC klasse I moleculen precies betrokken zijn bij herkenning door US2 en US11. Voor dit doel is een hele serie MHC klasse I moleculen gemaakt waarin telkens een ander domein is uitgewisseld tussen US2 of US11-gevoelige en ongevoelig MHC klasse I moleculen. Naast deze zogenaamde MHC klasse I chimeren zijn er nog een aantal mutanten gemaakt waarin slechts enkele bouwstenen (aminozuurresiduen) zijn veranderd.

De resultaten beschreven in **Hoofdstuk 2 en 4** laten duidelijk zien dat de cytoplasmatische staart van MHC klasse I bepalend kan zijn voor de gevoeligheid voor US11. Veranderingen in de staarten van de ongevoelige HLA-G en HLA-E moleculen zijn genoeg om ze volledig gevoelig te maken voor US11. De aan- of afwezigheid van een paar specifieke bouwstenen maakt hier een enorm verschil. Bij andere HLA-subgroepen levert dit een minder duidelijk beeld op. Zo blijkt uit proeven met HLA-A2-mutanten dat de samenstelling van de staart wel effect heeft op de

mate waarin de mutanten gevoelig zijn voor US11, maar dat dit niet de enige bepalende factor is voor deze gevoeligheid. Afhankelijk van de bouwsteen-samenstelling kunnen de in het ER gelegen domeinen van de zware ketens ook interacties met US11 aangaan. Welke individuele bouwstenen hierbij betrokken zijn moet nog nader worden bepaald.

Van US2 is er een kristalstructuur bekend, die in belangrijke mate laat zien hoe US2 een interactie aangaat met HLA-A2. Voor deze kristalstructuur is echter gebruik gemaakt van onvolledige versies van US2 en HLA-A2, die geen informatie bieden over eventuele interacties tussen de membraan-verankerde, of in het cytosol gelegen onderdelen van deze eiwitten. Om een goede indruk te krijgen welke domeinen in de zware ketens van klasse I moleculen bepalend zijn voor de effectiviteit van US2, is onderzocht of de staart- en membraandomeinen ook een bijdrage leveren.

Van HLA-G bestaat een variant waarin de transmembraan- en cytoplasmatische domeinen ontbreken. Deze oplosbare vorm van HLA-G wordt uiteindelijk door de cellen uitgescheiden. De resultaten in **Hoofdstuk 3** laten zien dat US2 deze HLA-G variant, in tegenstelling tot de membraan-gebonden vorm van HLA-G, niet kan afbreken. Naast de in het ER gelegen delen van de zware keten zijn er dus duidelijk ook andere domeinen essentieel voor een US2-gemedieerde afbraak. Maar zijn deze domeinen dan ook verantwoordelijk voor onderlinge verschillen in de mate waarin US2 de migratie van zware ketens naar het celoppervlak kan verhinderen? De studies in **Hoofdstuk 3 en 4** met verschillende MHC klasse I chimeren duiden erop dat dit niet het geval is: alleen de bouwsteensamenstelling van de in het ER gelegen delen van de zware ketens zijn uiteindelijk bepalend voor de mate waarin hun expressie op het celoppervlak door US2 wordt verhinderd.

De volgende stap was om te kijken of de gebieden die in HLA-A2 belangrijk zijn voor de interactie met US2, ook een rol spelen bij de selectie van andere HLA-typen die onder invloed van US2 kunnen worden afgebroken. Als de aminozuursequenties van diverse klasse I moleculen naast elkaar worden gelegd valt op dat er inderdaad verschillende residuen op deze posities kunnen voorkomen. Door precies deze aminozuren te veranderen in de US2-ongevoelige HLA-B7 en HLA-E moleculen, kon er met het in **Hoofdstuk 4** beschreven onderzoek worden aan-getoond, dat een verandering van slechts 1-4 residuen al een enorme invloed heeft op de oppervlakte-expressie van deze HLA moleculen in US2-positieve cellen. Op basis van gegevens uit een databank met MHC klasse I sequenties (bouw-tekeningen) kan er zo voor een groot deel van hen voorspeld worden of ze gevoelig zullen zijn voor US2.

In aanwezigheid van US2 of US11 worden MHC klasse I moleculen uit het ER membraan verwijderd en in het cytosol afgebroken. Het is nog steeds onduidelijk hoe dit proces verloopt. Het is wel bekend dat eiwitten die afgebroken dienen te worden eerst een soort etiket (ubiquitine) opgeplakt krijgen. Eiwitten die met een keten van deze ubiquitine moleculen zijn uitgerust worden vervolgens herkend door de eiwit-afbraakmachinerie in de cel, het proteasoom. In **Hoofdstuk 5** is beschreven in welke mate ubiquiti-nering een rol speelt bij het naar het cytosol trans-porteren van MHC klasse I moleculen in cellen waarin US11 actief is. De resultaten laten zien dat er in cellen met een defect 'etiketteerapparaat' geen transport van MHC klasse I moleculen uit het ER naar het cytosol plaats vindt. Ubiquitinering is dus erg belangrijk voor een goed verloop van het door US11 gemedieerde afbraakproces van MHC klasse I moleculen.

Het is onwaarschijnlijk dat US2 en US11 volledig zelfstandig te werk gaan; het is aannemelijker dat ze voor een belangrijk deel gebruik maken van bestaande cellulaire systemen. Zoals eerder vermeld worden MHC klasse I moleculen die niet aan de kwaliteitseisen voldoen ook de afbraakroute inge-stuurd (bijvoorbeeld omdat de assemblage met de lichte keten of antigeen fragmenten niet goed verloopt). Vernietiging van de MHC moleculen vindt in dit geval pas na een half uur tot een uur plaats, terwijl dit in cellen met US2 of US11 al binnen enkele minuten gebeurd.

Het is bekend welke chaperonne eiwitten de speci-fieke stappen van het assemblage proces van MHC klasse I faciliteren en het lijkt erop dat sommige van hen ook betrokken zijn bij de afbraak van deze moleculen. Om meer inzicht te krijgen in de manier waarop US2 en US11 te werk gaan, is onderzocht op welk moment US2 en US11 precies aangrijpen in dit assemblageproces. Voor dit in **Hoofdstuk 6** beschreven onderzoek is gebruik gemaakt van een cellijn waarin de lichte keten ontbreekt. Zonder deze lichte keten stopt het assemblage proces al in een vroeg stadium. De resultaten laten zien dat de virale eiwitten de zware ketens zowel in aan- als afwezigheid van de lichte keten prima kunnen laten

afbreken. De virale eiwitten kunnen dus in een heel vroeg stadium van het assemblage proces aangrijpen, op een moment waarop de chaperonne-eiwitten BiP, calnexin en Erp57 nog bij het assemblageproces zijn betrokken.
Een interessante bevinding werd gedaan toen de proeven werden uitgevoerd in aanwezigheid van een stof die het proteasoom (de al eerder genoemde afbraakmachine in het cytosol) blokkeert. Onder deze omstandigheden slaagt US11 er nauwelijks meer in om de zware ketens naar het cytosol te krijgen in de cellen zonder lichte ketens. Er is echter geen probleem als de lichte ketens normaal aanwezig zijn. US2 heeft onder geen van deze omstandigheden problemen om de afbraak van MHC klasse I moleculen in gang te zetten.
De transportroute naar het cytosol wordt ook geremd als de proteasomen geblokkeerd worden in de cellen waarin de vrije zware ketens op US2 / US11-onafhankelijke wijze worden afgebroken, bijvoorbeeld omdat zij niet aan de kwaliteitseisen voldoen doordat de lichte keten ontbreekt. Deze resultaten geven aan dat de zware ketens in US2- en US11-positive cellen op verschillende manieren naar de proteasomen worden gevoerd. Tevens lijkt er een overlap te bestaan in de route die gewoonlijk doorlopen wordt door afgekeurde MHC klasse I ketens en de route die door US11 in gang wordt gezet.

Al met al hebben de studies in dit proefschrift bijgedragen tot een beter inzicht in het type HLA moleculen dat onder invloed van US2 en US11 worden afgebroken, wat belangrijk is voor een juiste interpretatie van de effecten op de functie van T cellen en NK cellen. Daarnaast hebben de studies meer informatie verschaft over de mechanismen die door US2 en US11 gebruikt worden om klasse I moleculen vanuit het ER naar het cytosol te dirigeren, waar zij vervolgens worden afgebroken.

Publications

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